BOX SEQ - A

DOCKET NO. : ISIS-3455 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

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Serial No.: Not Yet Assigned **Group Art Unit:** Not Yet Assigned

Filing Date: Herewith **Examiner:** Not Yet Assigned

For: IDENTIFICATION OF GENETIC TARGETS FOR MODULATION BY OLIGONUCLEOTIDES AND GENERATION OF OLIGONUCLEOTIDES FOR GENE MODULATION

EXPRESS MAIL LABEL NO: EL066387979US
DATE OF DEPOSIT: April 13, 1999

Box ☐ Patent Application

☐ Provisional ☐ Design ☒ Sequence

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☐ divisional ☒ continuation-in-part of prior application number 09/067,638, filed April 28, 1998.

☐ A Provisional Patent Application under 37 C.F.R. 1.53(c).

☐ A Design Patent Application (submitted in duplicate).

Including the following:

☐ Provisional Application Cover Sheet.

☒ New or Revised Specification, including pages 1 to 131 containing:

☒ Specification

☒ Claims

☒ Abstract

☐ Substitute Specification, including Claims and Abstract.

☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.

☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.

☐ A copy of earlier application Serial No. _____ Filed _____, including Specification, Claims and Abstract (pages 1 - @@), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

☐ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application:
☐ is a continuation of ☐ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. _____filed _____
_____
_____
_____.

☐ Signed Statement attached deleting inventor(s) named in the prior application.

☐ A Preliminary Amendment.

☒ Twenty-four (24) Sheets of ☒ Formal ☐ Informal Drawings.

☐ Petition to Accept Photographic Drawings.

☐ Petition Fee

☒ An ☐ Executed ☒ Unexecuted Declaration or Oath and Power of Attorney.

☐ An Associate Power of Attorney.

☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to _____
_____.

☐ A Recordation Form Cover Sheet.

☐ Recordation Fee - $40.00.

☐ The prior application is assigned of record to _____

☐ Priority is claimed under 35 U.S.C. § 119 of Patent Application No. _____ filed _____ in _____ (country).

☐ A Certified Copy of each of the above applications for which priority is claimed:

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____ .

☐ An ☐ Executed or ☐ Copy of Executed Earlier Statement Claiming Small Entity

Status under 37 C.F.R. 1.9 and 1.27

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.

☒ Diskette Containing DNA/Amino Acid Sequence Information.

☒ Statement to Support Submission of DNA/Amino Acid Sequence Information.

☐ The computer readable form in this application _____, is identical with that filed in Application Serial Number _____, filed _____. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.

☐ Information Disclosure Statement.

- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☐ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: _____ .

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: _____

_____

_____ .

FEE CALCULATION:

☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

			SMALL ENTITY		NOT SMALL ENTITY	
			RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION			$75.00	$	$150.00	$
DESIGN APPLICATION			$155.00	$	$310.00	$
UTILITY APPLICATIONS BASE FEE			$380.00	$380.00	$760.00	$
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS						
	No. Filed	No. Extra				
TOTAL CLAIMS	111- 20 =	91	$9 each	$819.00	$18 each	$
INDEP. CLAIMS	45- 3 =	42	$39 each	$1638.00	$78 each	$
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			$130	$130	$260	$
ADDITIONAL FILING FEE				$0		$
TOTAL FILING FEE DUE				$2967.00		$

☒ A Check is enclosed in the amount of $ 2967.00 .

☐ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

- ☐ The foregoing amount due.
- ☐ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.
- ☐ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).
- ☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-identified application during the pendency of this application. The Commissioner is

further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: April 13, 1999

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IDENTIFICATION OF GENETIC TARGETS FOR MODULATION BY OLIGONUCLEOTIDES AND GENERATION OF OLIGONUCLEOTIDES FOR GENE MODULATION

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is a continuation-in-part of U.S. Serial No. 09/067,638 filed April 28, 1998, which claims priority to provisional application Serial No. 60/081,483 filed April 13, 1998, each of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates generally to the generation and identification of synthetic compounds having defined physical, chemical or bioactive properties. More particularly, the present invention relates to the automated generation of oligonucleotide compounds targeted to a given nucleic acid sequence via computer-based, iterative robotic synthesis of synthetic oligonucleotide compounds and robotic or robot-assisted analysis of the activities of such compounds. Information gathered from assays of such compounds is used to identify nucleic acid sequences that are tractable to a variety of nucleotide sequence-based technologies, for example, antisense drug discovery and target validation.

BACKGROUND OF THE INVENTION

1. Oligonucleotide Technology

Synthetic oligonucleotides of complementarity to targets are known to hybridize with particular, target nucleic acids in a sequence-specific manner. In one example, compounds complementary to the "sense" strand of nucleic acids that encode polypeptides, are referred to as "antisense oligonucleotides." A subset of such compounds may be capable of modulating the expression of a target nucleic acid; such synthetic compounds are described herein as "active oligonucleotide compounds."

Oligonucleotide compounds are commonly used *in vitro* as research reagents and diagnostic aids, and *in vivo* as therapeutic and bioactive agents. Oligonucleotide compounds can exert their effect by a variety of means. One such means takes advantage of an endogenous nuclease, such as RNase H in eukaryotes or RNase P in prokaryotes, to degrade the DNA/RNA hybrid formed between the oligonucleotide sequence and mRNA (Chiang *et al.*, *J. Biol. Chem.*, 1991, *266*, 18162; Forster *et al.*, *Science*, 1990, *249*, 783). Another means involves covalently linking of a synthetic moiety having nuclease activity to an oligonucleotide having an antisense sequence. This does not rely upon recruitment of an endogenous nuclease to modulate target activity. Synthetic moieties having nuclease activity include, but are not limited to, enzymatic RNAs, lanthanide ion complexes, and other reactive species. (Haseloff *et al.*, Nature, 1988, *334*, 585; Baker *et al.*, *J. Am. Chem. Soc.*, 1997, *119*, 8749).

Despite the advances made in utilizing antisense technology to date, it is still common to identify target sequences amenable to antisense technologies through an empirical approach (Szoka, *Nature Biotechnology*, 1997, *15*, 509). Accordingly, the need exists for systems and methods for efficiently and effectively identifying target nucleotide sequences that are suitable for antisense modulation. The present disclosure answers this need by providing systems and methods for automatically identifying such sequences via *in silico,* robotic or other automated means.

2. Identification of Active Oligonucleotide Compounds

Traditionally, new chemical entities with useful properties are generated by (1) identifying a chemical compound (called a "lead compound") with some desirable property or activity, (2) creating variants of the lead compound, and (3) evaluating the

property and activity of such variant compounds. The process has been called "SAR," i.e., structure activity relationship. Although "SAR" and its handmaiden, rational drug design, has been utilized with some degree of success, there are a number of limitations to these approaches to lead compound generation, particularly as it pertains to the discovery of bioactive oligonucleotide compounds. In attempting to use SAR with oligonucleotides, it has been recognized that RNA structure can inhibit duplex formation with antisense compounds, so much so that "moving" the target nucleotide sequence even a few bases can drastically decrease the activity of such compounds (Lima *et al.*, *Biochemistry*, 1992, *31*, 12055).

Heretofore, the preferred method of searching for lead antisense compounds has been the manual synthesis and analysis of such compounds. Consequently, a fundamental limitation of the conventional approach is its dependence upon the availability, number and cost of antisense compounds produced by manual, or at best semi-automated, means. Moreover, the assaying of such compounds has traditionally been performed by tedious manual techniques. Thus, the traditional approach to generating active antisense compounds is limited by the relatively high cost and long time required to synthesize and screen a relatively small number of candidate antisense compounds.

Accordingly, the need exists for systems and methods for efficiently and effectively generating new active antisense and other oligonucleotide compounds targeted to specific nucleic acid sequences. The present disclosure answers this need by providing systems and methods for automatically generating and screening active antisense compounds via robotic and other automated means.

3. Gene Function Analysis

Efforts such as the Human Genome Project are making an enormous amount of nucleotide sequence information available in a variety of forms, e.g., genomic sequences, cDNAs, expressed sequence tags (ESTs) and the like. This explosion of information has led one commentator to state that "genome scientists are producing more genes than they can put a function to" (Kahn, *Science*, 1995, *270*, 369). Although some approaches to this problem have been suggested, no solution has yet emerged. For example, methods of looking at gene expression in different disease states or stages of development only provide, at best, an association between a gene and a disease or stage of development

(Nowak, *Science*, 1995, *270*, 368). Another approach, looking at the proteins encoded by genes, is developing but "this approach is more complex and big obstacles remain" (Kahn, *Science*, 1995, *270*, 369). Furthermore, neither of these approaches allows one to directly utilize nucleotide sequence information to perform gene function analysis.

In contrast, antisense technology does allow for the direct utilization of nucleotide sequence information for gene function analysis. Once a target nucleic acid sequence has been selected, antisense sequences hybridizable to the sequence can be generated using techniques known in the art. Typically, a large number of candidate antisense oligonucleotides (ASOs) are synthesized having sequences that are more-or-less randomly spaced across the length of the target nucleic acid sequence (e.g., a "gene walk") and their ability to modulate the expression of the target nucleic acid is assayed. Cells or animals can then be treated with one or more active antisense oligonucleotides, and the resulting effects determined in order to determine the function(s) of the target gene. Although the practicality and value of this empirical approach to determining gene function has been acknowledged in the art, it has also been stated that this approach "is beyond the means of most laboratories and is not feasible when a new gene sequence is identified, but whose function and therapeutic potential are unknown" (Szoka, *Nature Biotechnology*, 1997, 15, 509).

Accordingly, the need exists for systems and methods for efficiently and effectively determining the function of a gene that is uncharacterized except that its nucleotide sequence, or a portion thereof, is known. The present disclosure answers this need by providing systems and methods for automatically generating active antisense compounds to a target nucleotide sequence via robotic means. Such active antisense compounds are contacted with cells, cell-free extracts, tissues or animals capable of expressing the gene of interest and subsequent biochemical or biological parameters are measured. The results are compared to those obtained from a control cell culture, cell-free extract, tissue or animal which has not been contacted with an active antisense compound in order to determine the function of the gene of interest.

4. Target Validation

Determining the nucleotide sequence of a gene is no longer an end unto itself; rather, it is "merely a means to an end. The critical next step is to validate the gene and its

[gene] product as a potential drug target" (Glasser, *Genetic Engineering News*, 1997, *17*, 1). This process, i.e., confirming that modulation of a gene that is suspected of being involved in a disease or disorder actually results in an effect that is consistent with a causal relationship between the gene and the disease or disorder, is known as target validation.

Efforts such as the Human Genome Project are yielding a vast number of complete or partial nucleotide sequences, many of which might correspond to or encode targets useful for new drug discovery efforts. The challenge represented by this plethora of information is how to use such nucleotide sequences to identify and rank valid targets for drug discovery. Antisense technology provides one means by which this might be accomplished; however, the many manual, labor-intensive and costly steps involved in traditional methods of developing active antisense compounds has limited their use in target validation (Szoka, *Nature Biotechnology*, 1997, *15*, 509). Nevertheless, the great target specificity that is characteristic of antisense compounds makes them ideal choices for target validation, especially when the functional roles of proteins that are highly related are being investigated (Albert *et al.*, *Trends in Pharm. Sci.*, 1994, *15*, 250).

Accordingly, the need exists for systems and methods for developing compounds efficiently and effectively that modulate a gene, wherein such compounds can be directly developed from nucleotide sequence information. Such compounds are needed to confirm that modulation of a gene that is thought to be involved in a disease or disorder will in fact cause an *in vitro* or *in vivo* effect indicative of the origin, development, spread or growth of the disease or disorder.

The present disclosure answers this need by providing systems and methods for automatically generating active oligonucleotide and other compounds, especially antisense compounds, to a target nucleotide sequence via robotic or other automated means. Such active compounds are contacted with a cell culture, cell-free extract, tissue or animal capable of expressing the gene of interest, and subsequent biochemical or biological parameters indicative of the potential gene product function are measured. These results are compared to those obtained with a control cell system, cell-free extract, tissue or animal which has not been contacted with an active antisense compound in order to determine whether or not modulation of the gene of interest affects a specific cellular function. The resulting active antisense compounds may be used as positive controls when

other, non antisense-based agents directed to the same target nucleic acid, or to its gene product, are screened.

It should be noted that embodiments of the invention drawn to gene function analysis and target validation have parameters that are shared with other embodiments of the invention, but also have unique parameters. For example, antisense drug discovery naturally requires that the toxicity of the antisense compounds be manageable, whereas, for gene function analysis or target validation, overt toxicity resulting from the antisense compounds is acceptable unless it interferes with the assay being used to evaluate the effects of treatment with such compounds.

U.S. Patent 5,563,036 to Peterson *et al.* describes systems and methods of screening for compounds that inhibit the binding of a transcription factor to a nucleic acid. In a preferred embodiment, an assay portion of the process is stated to be performed by a computer controlled robot.

U.S. Patent 5,708,158 to Hoey describes systems and methods for identifying pharmacological agents stated to be useful for diagnosing or treating a disease associated with a gene the expression of which is modulated by a human nuclear factor of activated T cells. The methods are stated to be particularly suited to high-thoughput screening wherein one or more steps of the process are performed by a computer controlled robot.

U.S. Patents 5,693,463 and 5,716,780 to Edwards *et al.* describe systems and methods for identifying non-oligonucleotide molecules that specifically bind to a DNA molecule based on their ability to compete with a DNA-binding protein that recognizes the DNA molecule.

U.S. Patents 5,463,564 and 5,684,711 to Agrafiotis et al. describe computer based iterative processes for generating chemical entities with defined physical, chemical and/or bioactive properties.

SUMMARY OF THE INVENTION

The present invention is directed to automated systems and methods for defining sets of compounds that modulate the expression of target nucleic acid sequences, and generating sets of oligonucleotides that modulate the expression of target nucleic acid sequences. The present invention is also directed to identifying nucleic acid sequences

amenable to antisense binding of oligonucleotides to those nucleic acid sequences by the systems and methods of the invention. For purposes of illustration, the present invention is described herein with respect to the production and identification of active antisense oligonucleotides; however, the present invention is not limited to this embodiment.

The present invention is directed to iterative processes for defining chemical compounds with prescribed sets of physical, chemical and/or biological properties, and to systems for implementing these processes. During each iteration of a process as contemplated herein, a target nucleic acid sequence is provided or selected, and a library of (candidate) virtual compounds is generated *in silico* (that is in a computer manipulatible and reliable form) according to defined criteria. A library of virtual compounds is generated. These virtual compounds are reviewed and compounds predicted to have particular desired properties are selected. The selected compounds are synthesized, preferably in a robotic, batchwise manner; and then they are robotically assayed for a desired physical, chemical or biological activity in order to identify compounds with the desired properties. Active compounds are, thus, generated and, at the same time, preferred sequences and regions of the target nucleic acid that are amenable to modulation are identified. The preferred compounds of the invention are oligonucleotides that bind to a target nucleic acid sequence.

In subsequent iterations of the process, second libraries of candidate compounds are generated and/or selected to give rise to a second virtual compound library. Through multiple iterations of the process, a library of target nucleic acid sequences that are tractable to modulation via binding of these compounds to the nucleic acid sequence are identified. Such modulation includes, but is not limited to, antisense technology, gene function analysis and target validation.

The present invention is also directed to processes for validating the function of a gene or the product of the gene comprising generating *in silico* a library of nucleobase sequences targeted to the gene and robotically assaying a plurality of synthetic compounds having at least some of the nucleobase sequences for effects on biological function.

Further features and advantages of the present invention, as well as the structure and operation of various embodiments of the present invention, are described in detail below with reference to the accompanying drawings. In the drawings, like reference

numbers indicate identical or functionally similar elements.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be described with reference to the accompanying drawings, wherein:

Figures 1 and 2 are a flow diagram of one method according to the present invention depicting the overall flow of data and materials among various elements of the invention.

Figure 3 is a flow diagram depicting the flow of data and materials among elements of step **200** of Figure 1.

Figures 4 and 5 are a flow diagram depicting the flow of data and materials among elements of step **300** of Figure 1.

Figure 6 is a flow diagram depicting the flow of data and materials among elements of step **306** of Figure 4.

Figure 7 is another flow diagram depicting the flow of data and materials among elements of step **306** of Figure 4.

Figure 8 is a another flow diagram depicting the flow of data and materials among elements of step **306** of Figure 4.

Figure 9 is a flow diagram depicting the flow of data and materials among elements of step **350** of Figure 5.

Figures 10 and 11 are flow diagrams depicting a logical analysis of data and materials among elements of step **400** of Figure 1.

Figure 12 is a flow diagram depicting the flow of data and materials among the elements of step **400** of Figure 1.

Figures 13 and 14 are flow diagrams depicting the flow of data and materials among elements of step **500** of Figure 1.

Figure 15 is a flow diagram depicting the flow of data and materials among elements of step **600** of Figure 1.

Figure 16 is a flow diagram depicting the flow of data and materials among elements of step **700** of Figure 1.

Figure 17 is a flow diagram depicting the flow of data and materials among the

elements of step **1100** of Figure 2.

Figure 18 is a block diagram showing the interconnecting of certain devices utilized in conjunction with a preferred method of the invention;

Figure 19 is a flow diagram showing a representation of data storage in a relational database utilized in conjunction with one method of the invention;

Figure 20 is a flow diagram depicting the flow of data and materials in effecting a preferred embodiment of the invention as set forth in Example 14;

Figure 21 is a flow diagram depicting the flow of data and materials in effecting a preferred embodiment of the invention as set forth in Example 15;

Figure 22 is a flow diagram depicting the flow of data and materials in effecting a preferred embodiment of the invention as set forth in Example 2;

Figure 23 is a pictorial elevation view of a preferred apparatus used to robotically synthesize oligonucleotides; and

Figure 24 is a pictorial plan view of an apparatus used to robotically synthesize oligonucleotides.

DETAILED DESCRIPTION OF THE INVENTION

Certain preferred methods of this invention are now described with reference to the flow diagram of Figures 1 and 2.

1. Target Nucleic Acid Selection. The target selection process, process step **100**, provides a target nucleotide sequence that is used to help guide subsequent steps of the process. It is generally desired to modulate the expression of the target nucleic acid for any of a variety of purposes, such as, e.g., drug discovery, target validation and/or gene function analysis.

One of the primary objectives of the target selection process, step **100**, is to identify molecular targets that represent significant therapeutic opportunities, to provide new and efficacious means of drug discovery and to determine the function of genes that are uncharacterized except for nucleotide sequence. To meet these objectives, genes are classified based upon specific sets of selection criteria.

One such set of selection criteria concerns the quantity and quality of target nucleotide sequence. There must be sufficient target nucleic acid sequence information

available for oligonucleotide design. Moreover, such information must be of sufficient quality to give rise to an acceptable level of confidence in the data to perform the methods described herein. Thus, the data must not contain too many missing or incorrect base entries. In the case of a target sequence that encodes a polypeptide, such errors can often be detected by virtually translating all three reading frames of the sense strand of the target sequence and confirming the presence of a continuous polypeptide sequence having predictable attributes, e.g., encoding a polypeptide of known size, or encoding a polypeptide that is about the same length as a homologous protein. In any event, only a very high frequency of sequence errors will frustrate the methods of the invention; most oligonucleotides to the target sequence will avoid such errors unless such errors occur frequently throughout the entire target sequence.

Another preferred criterion is that appropriate culturable cell lines or other source of reproducible genetic expression should be available. Such cell lines express, or can be induced to express, the gene comprising the target nucleic acid sequence. The oligonucleotide compounds generated by the process of the invention are assayed using such cell lines and, if such assaying is performed robotically, the cell line is preferably tractable to robotic manipulation such as by growth in 96 well plates. Those skilled in the art will recognize that if an appropriate cell line does not exist, it will nevertheless be possible to construct an appropriate cell line. For example, a cell line can be transfected with an expression vector comprising the target gene in order to generate an appropriate cell line for assay purposes.

For gene function analysis, it is possible to operate upon a genetic system having a lack of information regarding, or incomplete characterization of, the biological function(s) of the target nucleic acid or its gene product(s). This is a powerful agent of the invention. A target nucleic acid for gene function analysis might be absolutely uncharacterized, or might be thought to have a function based on minimal data or homology to another gene. By application of the process of the invention to such a target, active compounds that modulate the expression of the gene can be developed and applied to cells. The resulting cellular, biochemical or molecular biological responses are observed, and this information is used by those skilled in the art to elucidate the function of the target gene.

For target validation and drug discovery, another selection criterion is disease

association. Candidate target genes are placed into one of several broad categories of known or deduced disease association. Level 1 Targets are target nucleic acids for which there is a strong correlation with disease. This correlation can come from multiple scientific disciplines including, but not limited to, epidemiology, wherein frequencies of gene abnormalities are associated with disease incidence; molecular biology, wherein gene expression and function are associated with cellular events correlated with a disease; and biochemistry, wherein the *in vitro* activities of a gene product are associated with disease parameters. Because there is a strong therapeutic rationale for focusing on Level 1 Targets, these targets are most preferred for drug discovery and/or target validation.

Level 2 Targets are nucleic acid targets for which the combined epidemiological, molecular biological, and/or biochemical correlation with disease is not so clear as for Level 1. Level 3 Targets are targets for which there is little or no data to directly link the target with a disease process, but there is indirect evidence for such a link, i.e., homology with a Level 1 or Level 2 target nucleic acid sequence or with the gene product thereof. In order not to prejudice the target selection process, and to ensure that the maximum number of nucleic acids actually involved in the causation, potentiation, aggravation, spread, continuance or after-effects of disease states are investigated, it is preferred to examine a balanced mix of Level 1, 2 and 3 target nucleic acids.

In order to carry out drug discovery, experimental systems and reagents shall be available in order for one to evaluate the therapeutic potential of active compounds generated by the process of the invention. Such systems may be operable *in vitro* (e.g., *in vitro* models of cell:cell association) or *in vivo* (e.g., animal models of disease states). It is also desirable, but not obligatory, to have available animal model systems which can be used to evaluate drug pharmacology.

Candidate targets nucleic acids can also classified by biological processes. For example, programmed cell death ("apoptosis") has recently emerged as an important biological process that is perturbed in a wide variety of diseases. Accordingly, nucleic acids that encode factors that play a role in the apoptotic process are identified as candidate targets. Similarly, potential target nucleic acids can be classified as being involved in inflammation, autoimmune disorders, cancer, or other pathological or dysfunctional processes.

Moreover, genes can often be grouped into families based on sequence homology and biological function. Individual family members can act redundantly, or can provide specificity through diversity of interactions with downstream effectors, or through expression being restricted to specific cell types. When one member of a gene family is associated with a disease process then the rationale for targeting other members of the same family is reasonably strong. Therefore, members of such gene families are preferred target nucleic acids to which the methods and systems of the invention may be applied. Indeed, the potent specificity of antisense compounds for different gene family members makes the invention particularly suited for such targets (Albert *et al.*, *Trends Pharm. Sci.*, 1994, *15*, 250). Those skilled in the art will recognize that a partial or complete nucleotide sequence of such family members can be obtained using the polymerase chain reaction (PCR) and "universal" primers, i.e., primers designed to be common to all members of a given gene family.

PCR products generated from universal primers can be cloned and sequenced or directly sequenced using techniques known in the art. Thus, although nucleotide sequences from cloned DNAs, or from complementary DNAs (cDNAs) derived from mRNAs, may be used in the process of the invention, there is no requirement that the target nucleotide sequence be isolated from a cloned nucleic acid. Any nucleotide sequence, no matter how determined, of any nucleic acid, isolated or prepared in any fashion, may be used as a target nucleic acid in the process of the invention.

Furthermore, although polypeptide-encoding nucleic acids provide the target nucleotide sequences in one embodiment of the invention, other nucleic acids may be targeted as well. Thus, for example, the nucleotide sequences of structural or enzymatic RNAs may be utilized for drug discovery and/or target validation when such RNAs are associated with a disease state, or for gene function analysis when their biological role is not known.

2. Assembly of Target Nucleotide Sequence. Figure 3 is a block diagram detailing the steps of the target nucleotide sequence assembly process, process step **200** in acccordance with one embodiment of the invention. The oligonucleotide design process, process step **300**, is facilitated by the availability of accurate target sequence information. Because of limitations of automated genome sequencing technology, gene sequences are

often accumulated in fragments. Further, because individual genes are often being sequenced by independent laboratories using different sequencing strategies, sequence information corresponding to different fragments is often deposited in different databases. The target nucleic acid assembly process take advantage of computerized homology search algorithms and sequence fragment assembly algorithms to search available databases for related sequence information and incorporate available sequence information into the best possible representation of the target nucleic acid molecule, for example a RNA transcript. This representation is then used to design oligonucleotides, process step **300**, which can be tested for biological activity in process step **700**.

In the case of genes directing the synthesis of multiple transcripts, i.e. by alternative splicing, each distinct transcript is a unique target nucleic acid for purposes of step **300**. In one embodiment of the invention, if active compounds specific for a given transcript isoform are desired, the target nucleotide sequence is limited to those sequences that are unique to that transcript isoform. In another embodiment of the invention, if it is desired to modulate two or more transcript isoforms in concert, the target nucleotide sequence is limited to sequences that are shared between the two or more transcripts.

In the case of a polypeptide-encoding nucleic acid, it is generally preferred that full-length cDNA be used in the oligonucleotide design process step **300** (with full-length cDNA being defined as reading from the 5' cap to the poly A tail). Although full-length cDNA is preferred, it is possible to design oligonucleotides using partial sequence information. Therefore it is not necessary for the assembly process to generate a complete cDNA sequence. Further in some cases it may be desirable to design oligonucleotides targeting introns. In this case the process can be used to identify individual introns at process step **220**.

The process can be initiated by entering initial sequence information on a selected molecular target at process step **205**. In the case of a polypeptide-encoding nucleic acid, the full-length cDNA sequence is generally preferred for use in oligonucleotide design strategies at process step **300**. The first step is to determine if the initial sequence information represents the full-length cDNA, decision step **210**. In the case where the full-length cDNA sequence is available the process advances directly to the oligonucleotide design step **300**. When the full-length cDNA sequence is not available, databases are

searched at process step **212** for additional sequence information.

The algorithm preferably used in process steps **212** and **230** is BLAST (Altschul, *et al.*, *J. Mol. Biol.*, **1990**, *215*, 403), or "Gapped BLAST" (Altschul *et al.*, *Nucl. Acids Res.*, 1997, *25*, 3389). These are database search tools based on sequence homology used to identify related sequences in a sequence database. The BLAST search parameters are set to only identify closely related sequences. Some preferred databases searched by BLAST are a combination of public domain and proprietary databases. The databases, their contents, and sources are listed in Table 1.

Table 1: Database Sources of Target Sequences

Database	Contents	Source
NR	All non-redundant GenBank, EMBL, DDBJ and PDB sequences	National Center for Biotechnology Information at the National Institutes of Health
Month	All new or revised GenBank, EMBL, DDBJ and PDB sequences released in the last 30 days	National Center for Biotechnology Information at the National Institutes of Health
Dbest	Non-redundant database of GenBank, EMBL, DDBJ and EST divisions	National Center for Biotechnology Information at the National Institutes of Health
Dbsts	Non-redundant database of GenBank, EMBL, DDBJ and STS divisions	National Center for Biotechnology Information at the National Institutes of Health
Htgs	High throughput genomic sequences	National Center for Biotechnology Information at the National Institutes of Health

When genomic sequence information is available at decision step **215,** introns are removed and exons are assembled into continuous sequence representing the cDNA sequence in process step **220**. Exon assembly occurs using the Phragment Assembly Program "Phrap" (Copyright University of Washington Genome Center, Seattle, WA). The Phrap algorithm analyzes sets of overlapping sequences and assembles them into one

continuous sequence referred to as a "contig." The resulting contig is preferably used to search databases for additional sequence information at process step **230**. When genomic information is not available, the results of process step **212** are analyzed for individual exons at decision step **225**. Exons are frequently recorded individually in databases. If multiple complete exons are identified, they are prferably assembled into a contig using Phrap at process step **250**. If multiple complete exons are not identified at decision step **225,** then sequences can be analyzed for partial sequence information in decision step **228**. ESTs identified in the database dbEST are examples of such partial sequence information. If additional partial information is not found, then the process is advanced to process step **230** at decision step **228**. If partial sequence information is found in process **212** then that information is advanced to process step **230** via decision step **228**.

Process step **230**, decision step **240**, decision step **260** and process step **250** define a loop designed to extend iteratively the amount of sequence information available for targeting. At the end of each iteration of this loop, the results are analyzed in decision steps **240** and **260**. If no new information is found then the process advances at decision step **240** to process step **300**. If there is an unexpectedly large amount of sequence information identified, suggesting that the process moved outside the boundary of the gene into repetitive genomic sequence, then the process is preferably cycled back one iteration and that sequence is advanced at decision step **240** to process step **300**. If a small amount of new sequence information is identified, then the loop is iterated such as by taking the 100 most 5-prime (5') and 100 most 3-prime (3') bases and interating them through the BLAST homology search at process step **230**. New sequence information is added to the existing contig at process step **250**.

3. *In Silico* Generation of a Set of Nucleobase Sequences and Virtual Oligonucleotides.

For the following steps **300** and **400**, they may be performed in the order described below, i.e., step **300** before step **400**, or, in an alternative embodiment of the invention, step **400** before step **300**. In this alternate embodiment, each oligonucleotide chemistry is first assigned to each oligonucleotide sequence. Then, each combination of oligonucleotide chemistry and sequence is evaluated according to the parameters of step **300**. This embodiment has the desirable feature of taking into account the effect of

alternative oligonucleotide chemistries on such parameters. For example, substitution of 5-methyl cytosine (5MeC or m5c) for cytosine in an antisense compound may enhance the stability of a duplex formed between that compound and its target nucleic acid. Other oligonucleotide chemistries that enhance oligonucleotide:[target nucleic acid] duplexes are known in the art (see for example, Freier *et al.*, *Nucleic Acids Research*, 1997, *25*, 4429). As will be appreciated by those skilled in the art, different oligonucleotide chemistries may be preferred for different target nucleic acids. That is, the optimal oligonucleotide chemistry for binding to a target DNA might be suboptimal for binding to a target RNA having the same nucleotide sequence.

In effecting the process of the invention in the order step **300** before step **400** as seen in Figure 1, from a target nucleic acid sequence assembled at step **200**, a list of oligonucleotide sequences is generated as represented in the flowchart shown in Figures 4 and 5. In step **302**, the desired oligonucleotide length is chosen. In a preferred embodiment, oligonucleotide length is between from about 8 to about 30, more preferably from about 12 to about 25, nucleotides. In step **304**, all possible oligonucleotide sequences of the desired length capable of hybridizing to the target sequence obtained in step **200** are generated. In this step, a series of oligonucleotide sequences are generated, simply by determining the most 5' oligonucleotide possible and "walking" the target sequence in increments of one base until the 3' most oligonucleotide possible is reached.

In step **305**, a virtual oligonucleotide chemistry is applied to the nucleobase sequences of step **304** in order to yield a set of virtual oligonucleotides that can be evaluated *in silico*. Default virtual oligonucleotide chemistries include those that are well-characterized in terms of their physical and chemical properties, e.g., 2'-deoxyribonucleic acid having naturally occurring bases (A, T, C and G), unmodified sugar residues and a phosphodiester backbone.

4. *In Silico* Evaluation of Thermodynamic Properties of Virtual Oligonucleotides.

In step **306**, a series of thermodynamic, sequence, and homology scores are preferably calculated for each virtual oligonucleotide obtained from step **305**. Thermodynamic properties are calculated as represented in Figure 6. In step **308**, the desired thermodynamic properties are selected. As many or as few as desired can be

selected; optionally, none will be selected. The desired properties will typically include step **309**, calculation of the free energy of the target structure. If the oligonucleotide is a DNA molecule, then steps **310**, **312**, and **314** are performed. If the oligonucleotide is an RNA molecule, then steps **311**, **313** and **315** are performed. In both cases, these steps correspond to calculation of the free energy of intramolecular oligonucleotide interactions, intermolecular interactions and duplex formation. In addition, a free energy of oligonucleotide-target binding is preferably calculated at step **316**.

Other thermodynamic and kinetic properties may be calculated for oligonucleotides as represented at step **317**. Such other thermodynamic and kinetic properties may include melting temperatures, association rates, dissociation rates, or any other physical property that may be predictive of oligonucleotide activity.

The free energy of the target structure is defined as the free energy needed to disrupt any secondary structure in the target binding site of the targeted nucleic acid. This region includes any intra-target nucleotide base pairs that need to be disrupted in order for an oligonucleotide to bind to its complementary sequence. The effect of this localized disruption of secondary structure is to provide accessibility by the oligonucleotide. Such structures will include double helices, terminal unpaired and mismatched nucleotides, loops, including hairpin loops, bulge loops, internal loops and multibranch loops (Serra *et al.*, *Methods in Enzymology*, 1995, *259*, 242).

The intermolecular free energies refer to inherent energy due to the most stable structure formed by two oligonucleotides; such structures include dimer formation. Intermolecular free energies should also be taken into account when, for example, two or more oligonucleotides, of different sequence are to be administered to the same cell in an assay.

The intramolecular free energies refer to the energy needed to disrupt the most stable secondary structure within a single oligonucleotide. Such structures include, for example, hairpin loops, bulges and internal loops. The degree of intramolecular base pairing is indicative of the energy needed to disrupt such base pairing.

The free energy of duplex formation is the free energy of denatured oligonucleotide binding to its denatured target sequence. The oligonucleotide-target binding is the total binding involved, and includes the energies involved in opening up

intra- and inter- molecular oligonucleotide structures, opening up target structure, and duplex formation.

The most stable RNA structure is predicted based on nearest neighbor analysis (Xia, T., *et al., Biochemistry*, 1998, *37*, 14719-14735; Serra *et al.*, *Methods in Enzymology*, 1995, *259*, 242). This analysis is based on the assumption that stability of a given base pair is determined by the adjacent base pairs. For each possible nearest neighbor combination, thermodynamic properties have been determined and are provided. For double helical regions, two additional factors need to be considered, an entropy change required to initiate a helix and a entropy change associated with self-complementary strands only. Thus, the free energy of a duplex can be calculated using the equation:

$$\Delta G^\circ_T = \Delta H^\circ - T\Delta S^\circ$$

where:

ΔG is the free energy of duplex formation,

ΔH is the enthalpy change for each nearest neighbor,

ΔS is the entropy change for each nearest neighbor, and T is temperature.

The ΔH and ΔS for each possible nearest neighbor combination have been experimentally determined. These letter values are often available in published tables. For terminal unpaired and mismatched nucleotides, enthalpy and entropy measurements for each possible nucleotide combination are also available in published tables. Such results are added directly to values determined for duplex formation. For loops, while the available data is not as complete or accurate as for base pairing, one known model determines the free energy of loop formation as the sum of free energy based on loop size, the closing base pair, the interactions between the first mismatch of the loop with the closing base pair, and additional factors including being closed by AU or UA or a first mismatch of GA or UU. Such equations may also be used for oligoribonucleotide-target RNA interactions.

The stability of DNA duplexes is used in the case of intra- or intermolecular oligodeoxyribonucleotide interactions. DNA duplex stability is calculated using similar equations as RNA stability, except experimentally determined values differ between nearest neighbors in DNA and RNA and helix initiation tends to be more favorable in

DNA than in RNA (SantaLucia *et al.*, *Biochemistry*, 1996, *35*, 3555).

Additional thermodynamic parameters are used in the case of RNA/DNA hybrid duplexes. This would be the case for an RNA target and oligodeoxynucleotide. Such parameters were determined by Sugimoto *et al.* (*Biochemistry*, 1995, *34*, 11211). In addition to values for nearest neighbors, differences were seen for values for enthalpy of helix initiation.

5. *In Silico* Evaluation of Target Accessibility

Target accessibility is believed to be an important consideration in selecting oligonucleotides. Such a target site will possess minimal secondary structure and thus, will require minimal energy to disrupt such structure. In addition, secondary structure in oligonucleotides, whether inter- or intra-molecular, is undesirable due to the energy required to disrupt such structures. Oligonucleotide-target binding is dependent on both these factors. It is desirable to minimize the contributions of secondary structure based on these factors. The other contribution to oligonucleotide-target binding is binding affinity. Favorable binding affinities based on tighter base pairing at the target site is desirable.

Following the calculation of thermodynamic properties ending at step **317**, the desired sequence properties to be scored are selected at step **324**. As many or as few as desired can be selected; optionally, none will be selected. These properties include the number of strings of four guanosine residues in a row at step **325** or three guanosine in a row at step **326**, the length of the longest string of adenosines at step **327**, cytidines at step **328** or uridines or thymidines at step **329**, the length of the longest string of purines at step **330** or pyrimidine at step **331**, the percent composition of adenosine at step **332**, cytidine at step **333**, guanosine at step **334** or uridines or thymidines at step **335**, the percent composition of purines at step **336** or pyrimidines at step **337**, the number of CG dinucleotide repeats at step **338**, CA dinucleotide repeats at step **339** or UA or TA dinucleotide repeats at step **340**. In addition, other sequence properties may be used as found to be relevant and predictive of antisense efficacy, as represented at step **341**.

These sequence properties may be important in predicting oligonucleotide activity, or lack thereof. For example, U.S. Patent 5,523,389 discloses oligonucleotides containing stretches of three or four guanosine residues in a row. Oligonucleotides having such sequences may act in a sequence-independent manner. For an antisense approach, such a

mechanism is not usually desired. In addition, high numbers of dinucleotide repeats may be indicative of low complexity regions which may be present in large numbers of unrelated genes. Unequal base composition, for example, 90% adenosine, can also give non-specific effects. From a practical standpoint, it may be desirable to remove oligonucleotides that possess long stretches of other nucleotides due to synthesis considerations. Other sequences properties, either listed above or later found to be of predictive value may be used to select oligonucleotide sequences.

Following step **341**, the homology scores to be calculated are selected in step **342**. Homology to nucleic acids encoding protein isoforms of the target, as represented at step **343**, may be desired. For example, oligonucleotides specific for an isoform of protein kinase C can be selected. Also, oligonucleotides can be selected to target multiple isoforms of such genes. Homology to analogous target sequences, as represented at step **344**, may also be desired. For example, an oligonucleotide can be selected to a region common to both humans and mice to facilitate testing of the oligonucleotide in both species. Homology to splice variants of the target nucleic acid, as represented at step **345**, may be desired. In addition, it may be desirable to determine homology to other sequence variants as necessary, as represented in step **346**.

Following step **346**, from which scores were obtained in each selected parameter, a desired range is selected to select the most promising oligonucleotides, as represented at step **347**. Typically, only several parameters will be used to select oligonucleotide sequences. As structure prediction improves, additional parameters may be used. Once the desired score ranges are chosen, a list of all oligonucleotides having parameters falling within those ranges will be generated, as represented at step **348**.

6. Targeting Oligonucleotides to Functional Regions of a Nucleic Acid.

It may be desirable to target oligonucleotide sequences to specific functional regions of the target nucleic acid. A decision is made whether to target such regions, as represented in decision step **349**. If it is desired to target functional regions then process step **350** occurs as seen in greater detail in Figure 9. If it is not desired then the process proceeds to step **375**.

In step **350**, as seen in Figure 9, the desired functional regions are selected. Such regions include the transcription start site or 5' cap at step **353**, the 5' untranslated region

at step **354**, the start codon at step **355**, the coding region at step **356**, the stop codon at step **357**, the 3' untranslated region at step **358**, 5' splice sites at step **359** or 3' splice sites at step **360**, specific exons at step **361** or specific introns at step **362**, mRNA stabilization signal at step **363**, mRNA destabilization signal at step **364**, poly-adenylation signal at step **365**, poly-A addition site at step **366**, poly-A tail at step **367**, or the gene sequence 5' of known pre-mRNA at step **368**. In addition, additional functional sites may be selected, as represented at step **369**.

Many functional regions are important to the proper processing of the gene and are attractive targets for antisense approaches. For example, the AUG start codon is commonly targeted because it is necessary to initiate translation. In addition, splice sites are thought to be attractive targets because these regions are important for processing of the mRNA. Other known sites may be more accessible because of interactions with protein factors or other regulatory molecules.

After the desired functional regions are selected and determined, then a subset of all previously selected oligonucleotides are selected based on hybridization to only those desired functional regions, as represented by step **370**.

7. Uniform Distribution of Oligonucleotides.

Whether or not targeting functional sites is desired, a large number of oligonucleotide sequences may result from the process thus far. In order to reduce the number of oligonucleotide sequences to a manageable number, a decision is made whether to uniformly distribute selected oligonucleotides along the target, as represented in step **375**. A uniform distribution of oligonucleotide sequences will aim to provide complete coverage throughout the complete target nucleic acid or the selected functional regions. A computer-based program is used to automate the distribution of sequences, as represented in step **380**. Such a program factors in parameters such as length of the target nucleic acid, total number of oligonucleotide sequences desired, oligonucleotide sequences per unit length, number of oligonucleotide sequences per functional region. Manual selection of oligonucleotide sequences is also provided for by step **385**. In some cases, it may be desirable to manually select oligonucleotide sequences. For example, it may be useful to determine the effect of small base shifts on activity. Once the desired number of oligonucleotide sequences is obtained either from step **380** or step **385**, then these

oligonucleotide sequences are passed onto step **400** of the process, where oligonucleotide chemistries are assigned.

8. Assignment of Actual Oligonucleotide Chemistry.

Once a set of select nucleobase sequences has been generated according to the preceding process and decision steps, actual oligonucleotide chemistry is assigned to the sequences. An "actual oligonucleotide chemistry" or simply "chemistry" is a chemical motif that is common to a particular set of robotically synthesized oligonucleotide compounds. Preferred chemistries include, but are not limited to, oligonucleotides in which every linkage is a phosphorothioate linkage, and chimeric oligonucleotides in which a defined number of 5' and/or 3' terminal residues have a 2'-methoxyethoxy modification.

Chemistries can be assigned to the nucleobase sequences during general procedure step **400** (Figure 1). The logical basis for chemistry assignment is illustrated in Figures 10 and 11 and an iterative routine for stepping through an oligonucleotide nucleoside by nucleoside is illustrated in Figure 12. Chemistry assignment can be effected by assignment directly into a word processing program, via an interactive word processing program or via automated programs and devices. In each of these instances, the output file is selected to be in a format that can serve as an input file to automated synthesis devices.

9. Oligonucleotide Compounds.

In the context of this invention, in reference to oligonucleotides, the term "oligonucleotide" is used to refer to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. Thus this term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms, i.e., phosphodiester linked A, C, G, T and U nucleosides, because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

The oligonucleotide compounds in accordance with this invention can be of various lengths depending on various parameters, including but not limited to those discussed above in reference to the selection criteria of general procedure **300**. For use as

antisense oligonucleotides compounds of the invention preferably are from about 8 to about 30 nucleobases in length (i.e. from about 8 to about 30 linked nucleosides). Particularly preferred are antisense oligonucleotides comprising from about 12 to about 25 nucleobases. A discussion of antisense oligonucleotides and some desirable modifications can be found in De Mesmaeker *et al.*, *Acc. Chem. Res.*, 1995, *28*, 366. Other lengths of oligonucleotides might be selected for non-antisense targeting strategies, for instance using the oligonucleotides as ribozymes. Such ribozymes normally require oligonucleotides of longer length as is known in the art.

A nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a normal (where normal is defined as being found in RNA and DNA) pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Specific examples of preferred oligonucleotides useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

10. Selection of Oligonucleotide Chemistries.

In a general logic scheme as illustrated in Figures 10 and 11, for each nucleoside position, the user or automated device is interrogated first for a base assignment, followed

by a sugar assignment, a linker assignment and finally a conjugate assignment. Thus for each nucleoside, at process step **410** a base is selected. In selecting the base, base chemistry 1 can be selected at process step **412** or one or more alternative bases are selected at process steps **414**, **416** and **418**. After base selection is effected, the sugar portion of the nucleoside is selected. Thus for each nucleoside, at process step **420** a sugar is selected that together with the select base will complete the nucleoside. In selecting the sugar, sugar chemistry 1 can be selected at process **422** or one or more alternative sugars are selected at process steps **424**, **426** and **428**. For each two adjacent nucleoside units, at process step **430**, the internucleoside linker is selected. The linker chemistry for the internucleoside linker can be linker chemistry 1 selected at process step **432** or one or more alternative internucleoside linker chemistries are selected at process steps **434**, **436** and **438**.

In addition to the base, sugar and internucleoside linkage, at each nucleoside position, one or more conjugate groups can be attached to the oligonucleotide via attachment to the nucleoside or attachment to the internucleoside linkage. The addition of a conjugate group is integrated at process step **440** and the assignment of the conjugate group is effected at process step **450**.

For illustrative purposes in Figures 10 and 11, for each of the bases, the sugars, the internucleoside linkers, or the conjugates, chemistries 1 though n are illustrated. As described in this specification, it is understood that the number of alternate chemistries between chemistry 1 and alternative chemistry n, for each of the bases, the sugars, the internucleoside linkages and the conjugates, is variable and includes, but is not limited to, each of the specific alternative bases, sugar, internucleoside linkers and conjugates identified in this specification as well as equivalents known in the art.

Utilizing the logic as described in conjunction with Figures 10 and 11, chemistry is assigned, as is shown in Figure 12, to the list of oligonucleotides from general procedure **300**. In assigning chemistries to the oligonucleotides in this list, a pointer can be set at process step **452** to the first oligonucleotide in the list and at step **453** to the first nucleotide of that first oligonucleotide. The base chemistry is selected at step **410**, as described above, the sugar chemistry is selected at step **420**, also as described above, followed by selection of the internucleoside linkage at step **430**, also as described above.

At decision **440**, the process branches depending on whether a conjugate will be added at the current nucleotide position. If a conjugate is desired, the conjugate is selected at step **450**, also as described above.

Whether or not a conjugate was added at decision step **440**, an inquiry is made at decision step **454**. This inquiry asks if the pointer resides at the last nucleotide in the current oligonucleotide. If the result at decision step **454** is "No," the pointer is moved to the next nucleotide in the current oligonucleotide and the loop including steps **410**, **420**, **430**, **440** and **454** is repeated. This loop is reiterated until the result at decision step **454** is "Yes."

When the result at decision step **454** is "Yes," a query is made at decision step **460** concerning the location of the pointer in the list of oligonucleotides. If the pointer is not at the last oligonucleotide of the list, the "No" path of the decision step **460** is followed and the pointer is moved to the first nucleotide of the next oligonucleotide in the list at process step **458**. With the pointer set to the next oligonucleotide in the list, the loop that starts at process steps **453** is reiterated. When the result at decision step **460** is "Yes," chemistry has been assigned to all of the nucleotides in the list of oligonucleotides.

11. Description of Oligonucleotide Chemistries.

As is illustrated in Figure 10, for each nucleoside of an oligonucleotide, chemistry selection includes selection of the base forming the nucleoside from a large palette of different base units available. These may be "modified" or "natural" bases (also reference herein as nucleobases) including the natural purine bases adenine (A) and guanine (G), and the natural pyrimidine bases thymine (T), cytosine (C) and uracil (U). They further can include modified nucleobases including other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo uracils and cytosines particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and

3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie, International Edition*, 1991, *30*, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred for selection as the base. These are particularly useful when combined with a 2'-O-methoxyethyl sugar modifications, described below.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent 3,687,808, as well as U.S. Patents 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, each of which is incorporated herein by reference in its entirety. Reference is also made to allowed United States patent application 08/762,488, filed on December 10, 1996, commonly owned with the present application and which is incorporated herein by reference in its entirety.

In selecting the base for any particular nucleoside of an oligonucleotide, consideration is first given to the need of a base for a particular specificity for hybridization to an opposing strand of a particular target. Thus if an "A" base is required, adenine might be selected however other alternative bases that can effect hybridization in a manner mimicking an "A" base such as 2-aminoadenine might be selected should other consideration, e.g., stronger hybridization (relative to hybridization achieved with adenine), be desired.

As is illustrated in Figure 10, for each nucleoside of an oligonucleotide, chemistry selection includes selection of the sugar forming the nucleoside from a large palette of different sugar or sugar surrogate units available. These may be modified sugar groups, for instance sugars containing one or more substituent groups. Preferred substituent groups comprise the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; or O, S- or N-alkynyl; wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred substituent groups comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl), 2'-O-methoxyethyl, or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, *78*, 486) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylamino oxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in co-owned United States patent application Serial Number 09/016,520, filed on January 30, 1998, which is incorporated herein by reference in its entirety.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the sugar group, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. The nucleosides of the oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811;

5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the present application, each of which is incorporated herein by reference in its entirety, together with allowed United States patent application 08/468,037, filed on June 5, 1995, which is commonly owned with the present application and which is incorporated herein by reference in its entirety.

As is illustrated in Figure 10, for each adjacent pair of nucleosides of an oligonucleotide, chemistry selection includes selection of the internucleoside linkage. These internucleoside linkages are also referred to as linkers, backbones or oligonucleotide backbones. For forming these nucleoside linkages, a palette of different internucleoside linkages or backbones is available. These include modified oligonucleotide backbones, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalklyphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus containing linkages include, but are not limited to, U.S. Patents 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; and 5,697,248, certain of which are commonly owned with this application, each of which is incorporated herein by reference in its entirety.

Preferred internucleoside linkages for oligonucleotides that do not include a phosphorus atom therein, i.e., for oligonucleosides, have backbones that are formed by short chain alkyl or cycloalkyl intersugar linkages, mixed heteroatom and alkyl or cycloalkyl intersugar linkages, or one or more short chain heteroatomic or heterocyclic intersugar linkages. These include those having morpholino linkages (formed in part from

the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patents 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, each of which is incorporated herein by reference in its entirety.

In other preferred oligonucleotides, i.e., oligonucleotide mimetics, both the sugar and the intersugar linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-phosphate backbone of an oligonucleotide is replaced with an amide-containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is incorporated herein by reference in its entirety. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, 1991, *254*, 1497.

For the internucleoside linkages, the most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$,- $CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ (wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$) of the above referenced U.S. patent 5,489,677, and the amide

backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent 5,034,506.

In attaching a conjugate group to one or more nucleosides or internucleoside linkages of an oligonucleotide, various properties of the oligonucleotide are modified. Thus modification of the oligonucleotides of the invention to chemically link one or more moieties or conjugates to the oligonucleotide are intended to enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al., Proc. Natl. Acad. Sci. USA*, 1989, *86*, 6553), cholic acid (Manoharan *et al., Bioorg. Med. Chem. Let.*, 1994, *4*, 1053), a thioether, e.g., hexyl-*S*-tritylthiol (Manoharan *et al., Ann. N.Y. Acad. Sci.*, 1992, *660*, 306; Manoharan *et al., Bioorg. Med. Chem. Let.*, 1993, *3*, 2765), a thiocholesterol (Oberhauser *et al., Nucl. Acids Res.*, 1992, *20*, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al., EMBO J.*, 1991, *10*, 111; Kabanov *et al., FEBS Lett.*, 1990, *259*, 327; Svinarchuk *et al., Biochimie*, 1993, *75*, 49), a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al., Tetrahedron Lett.*, 1995, *36*, 3651; Shea *et al., Nucl. Acids Res.*, 1990, *18*, 3777), a polyamine or a polyethylene glycol chain (Manoharan *et al., Nucleosides & Nucleotides*, 1995, *14*, 969), or adamantane acetic acid (Manoharan *et al., Tetrahedron Lett.*, 1995, *36*, 3651), a palmityl moiety (Mishra *et al., Biochim. Biophys. Acta*, 1995, *1264*, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al., J. Pharmacol. Exp. Ther.*, 1996, *277*, 923).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patents 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923;

5,599,928 and 5,688,941, certain of which are commonly owned with the present application, and each of which is herein incorporated by reference in its entirety.

12. Chimeric Compounds.

It is not necessary for all positions in a given compound to be uniformly modified. In fact, more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes compounds which are chimeric compounds. "Chimeric" compounds or "chimeras," in the context of this invention, are compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids.

By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures representing the union of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as "hybrids" or "gapmers". Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patents 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned with the present application and each of

which is incorporated herein by reference in its entirety, together with commonly owned and allowed United States patent application serial number 08/465,880, filed on June 6, 1995, which is incorporated herein by reference in its entirety.

13. Description of Automated Oligonucleotide Synthesis.

In the next step of the overall process (illustrated in Figures 1 and 2), oligonucleotides are synthesized on an automated synthesizer. Although many devices may be employed, the synthesizer is preferably a variation of the synthesizer described in United States patents 5,472,672 and 5,529,756, each of which is incorporated herein by reference in its entirety. The synthesizer described in those patents is modified to include movement in along the Y axis in addition to movement along the X axis. As so modified, a 96-well array of compounds can be synthesized by the synthesizer. The synthesizer further includes temperature control and the ability to maintain an inert atmosphere during all phases of synthesis. The reagent array delivery format employs orthogonal X-axis motion of a matrix of reaction vessels and Y-axis motion of an array of reagents. Each reagent has its own dedicated plumbing system to eliminate the possibility of cross-contamination of reagents and line flushing and/or pipette washing. This in combined with a high delivery speed obtained with a reagent mapping system allows for the extremely rapid delivery of reagents. This further allows long and complex reaction sequences to be performed in an efficient and facile manner.

The software that operates the synthesizer allows the straightforward programming of the parallel synthesis of a large number of compounds. The software utilizes a general synthetic procedure in the form of a command (.cmd) file, which calls upon certain reagents to be added to certain wells *via* lookup in a sequence (.seq) file. The bottle position, flow rate, and concentration of each reagent is stored in a lookup table (.tab) file. Thus, once any synthetic method has been outlined, a plate of compounds is made by permutating a set of reagents, and writing the resulting output to a text file. The text file is input directly into the synthesizer and used for the synthesis of the plate of compounds. The synthesizer is interfaced with a relational database allowing data output related to the synthesized compounds to be registered in a highly efficient manner.

Building of the .seq, .cmd and .tab files is illustrated in Figure 13. Thus as a part of the general oligonucleotide synthesis procedure **500**, for each linker chemistry at

process step **502**, a synthesis file, i.e., a .cmd file, is built at process step **504**. This file can be built fresh to reflect a completely new set of machine commands reflecting a set of chemical synthesis steps or it can modify an existing file stored at process step **504** by editing that stored file in process step **508**. The .cmd files are built using a word processor and a command set of instructions as outlined below.

It will be appreciated that the preparation of control software and data files is within the routine skill of persons skilled in annotated nucleotide synthesis. The same will depend upon the hardware employed, the chemistries adopted and the design paradigm selected by the operator.

In a like manner to the building the .cmd files, .tab files are built to reflect the necessary reagents used in the automatic synthesizer for the particular chemistries that have been selected for the linkages, bases, sugars and conjugate chemistries. Thus for each of a set of these chemistries at process step **510**, a .tab file is built at process step **512** and stored at process step **514**. As with the .cmd files, an existing .tab file can be edited at process step **516**.

Both the .cmd files and the .tab files are linked together at process step **518** and stored for later retrieval in an appropriate sample database **520**. Linking can be as simple as using like file names to associate a .cmd file to its appropriate .tab file, e.g., synthesis_1.cmd is linked to synthesis_1.tab by use of the same preamble in their names.

The automated, multi-well parallel array synthesizer employs a reagent array delivery format, in which each reagent utilized has a dedicated plumbing system. As seen in Figures 23 and 24, an inert atmosphere **522** is maintained during all phases of a synthesis. Temperature is controlled *via* a thermal transfer plate **524**, which holds an injection molded reaction block **526**. The reaction plate assembly slides in the X-axis direction, while for example eight nozzle blocks (**528**, **530**, **532**, **534**, **536**, **538**, **540** and **542**) holding the reagent lines slide in the Y-axis direction, allowing for the extremely rapid delivery of any of 64 reagents to 96 wells. In addition, there are for example, six banks of fixed nozzle blocks (**544**, **546**, **548**, **550**, **552** and **554**) which deliver the same reagent or solvent to eight wells at once, for a total of 72 possible reagents.

In synthesizing oligonucleotides for screening, the target reaction vessels, a 96 well plate **556** (a 2-dimensional array), moves in one direction along the X axis, while the

series of independently controlled reagent delivery nozzles (**528**, **530**, **532**, **534**, **536**, **538**, **540** and **542**) move along the Y-axis relative to the reaction vessel **558**. As the reaction plate **556** and reagent nozzles (**528**, **530**, **532**, **534**, **536**, **538**, **540** and **542**) can be moved independently at the same time, this arrangement facilitates the extremely rapid delivery of up to 72 reagents independently to each of the 96 reaction vessel wells.

The system software allows the straightforward programming of the synthesis of a large number of compounds by supplying the general synthetic procedure in the form of the command file to call upon certain reagents to be added to specific wells *via* lookup in the sequence file with the bottle position, flow rate, and concentration of each reagent being stored in the separate reagent table file. Compounds can be synthesized on various scales. For oligonucleotides, a 200 nmole scale is typically selected while for other compounds larger scales, as for example a 10 μmole scale (3-5 mg), might be utilized. The resulting crude compounds are generally >80% pure, and are utilized directly for high throughput screening assays. Alternatively, prior to use the plates can be subjected to quality control (see general procedure 600 and Example 9) to ascertain their exact purity. Use of the synthesizer results in a very efficient means for the parallel synthesis of compounds for screening.

The software inputs accept tab delimited text files (as discussed above for file **504** and **512**) from any text editor. A typical command file, a .cmd file, is shown in Example 3 at Table 2. Typical sequence files, .seq files, are shown in Example 3 at Tables 3 and 4 (.SEQ file), and a typical reagent file, a .tab file, is shown in Example 3 at Table 5. Table 3 illustrates the sequence file for an oligonucleotide having 2'-deoxy nucleotides at each position with a phosphorothioate backbone throughout. Table 4 illustrates the sequence file for an oligonucleotide, again having a phosphorothioate backbone throughout, however, certain modified nucleoside are utilized in portions of the oligonucleotide. As shown in this table, 2'-O-(2-methoxyethyl) modified nucleosides are utilized in a first region (a wing) of the oligonucleotide, followed by a second region (a gap) of 2'-deoxy nucleotides and finally a third region (a further wing) that has the same chemistry as the first region. Typically some of the wells of the 96 well plate **556** may be left empty (depending on the number of oligonucleotides to be made during an individual synthesis) or some of the wells may have oligonucleotides that will serve as standards for comparison

or analytical purposes.

Prior to loading reagents, moisture sensitive reagent lines are purged with argon at **522** for 20 minutes. Reagents are dissolved to appropriate concentrations and installed on the synthesizer. Large bottles, collectively identified as **558** in Figure 23 (containing 8 delivery lines) are used for wash solvents and the delivery of general activators, trityl group cleaving reagents and other reagents that may be used in multiple wells during any particular synthesis. Small septa bottles, collectively identified as **560** in Figure 23, are utilized to contain individual nucleotide amidite precursor compounds. This allows for anhydrous preparation and efficient installation of multiple reagents by using needles to pressurize the bottle, and as a delivery path. After all reagents are installed, the lines are primed with reagent, flow rates measured, then entered into the reagent table (.tab file). A dry resin loaded plate is removed from vacuum and installed in the machine for the synthesis.

The modified 96 well polypropylene plate **556** is utilized as the reaction vessel. The working volume in each well is approximately 700 μl. The bottom of each well is provided with a pressed-fit 20 μm polypropylene frit and a long capillary exit into a lower collection chamber as is illustrated in Figure 5 of the above referenced United States Patent 5,372,672. The solid support for use in holding the growing oligonucleotide during synthesis is loaded into the wells of the synthesis plate **556** by pipetting the desired volume of a balanced density slurry of the support suspended in an appropriate solvent, typically an acetonitrile-methylene chloride mixture. Reactions can be run on various scales as for instance the above noted 200 nmole and 10 μmol scales. For oligonucleotide synthesis a CPG support is preferred, however other medium loading polystyrene-PEG supports such as TENTAGEL™ or ARGOGEL™ can also be used.

As seen in Figure 24, the synthesis plate is transported back and forth in the X-direction under an array of 8 moveable banks (**530**, **532**, **534**, **536**, **538**, **540**, **542** and **544**) of 8 nozzles (64 total) in the Y-direction, and 6 banks (**544**, **546**, **548**, **550**, **552** and **554**) of 48 fixed nozzles, so that each well can receive the appropriate amounts of reagents and/or solvents from any reservoir (large bottle or smaller septa bottle). A sliding balloon-type seal **562** surrounds this nozzle array and joins it to the reaction plate headspace **564**. A slow sweep of nitrogen or argon **522** at ambient pressure across the plate headspace is used

to preserve an anhydrous environment.

The liquid contents in each well do not drip out until the headspace pressure exceeds the capillary forces on the liquid in the exit nozzle. A slight positive pressure in the lower collection chamber can be added to eliminate residual slow leakage from filled wells, or to effect agitation by bubbling inert gas through the suspension. In order to empty the wells, the headspace gas outlet valve is closed and the internal pressure raised to about 2 psi. Normally, liquid contents are blown directly to waste **566**. However, a 96 well microtiter plate can be inserted into the lower chamber beneath the synthesis plate in order to collect the individual well eluents for spectrophotometric monitoring (trityl, etc.) of reaction progress and yield.

The basic plumbing scheme for the machine is the gas-pressurized delivery of reagents. Each reagent is delivered to the synthesis plate through a dedicated supply line, collectively identified at **568**, solenoid valve collectively identified at **570** and nozzle, collectively identified at **572**. Reagents never cross paths until they reach the reaction well. Thus, no line needs to be washed or flushed prior to its next use and there is no possibility of cross-contamination of reagents. The liquid delivery velocity is sufficiently energetic to thoroughly mix the contents within a well to form a homogeneous solution, even when employing solutions having drastically different densities. With this mixing, once reactants are in homogeneous solution, diffusion carries the individual components into and out of the solid support matrix where the desired reaction takes place. Each reagent reservoir can be plumbed to either a single nozzle or any combination of up to 8 nozzles. Each nozzle is also provided with a concentric nozzle washer to wash the outside of the delivery nozzles in order to eliminate problems of crystallized reactant buildup due to slow evaporation of solvent at the tips of the nozzles. The nozzles and supply lines can be primed into a set of dummy wells directly to waste at any time.

The entire plumbing system is fabricated with teflon tubing, and reagent reservoirs are accessed *via* syringe needle/septa or direct connection into the higher capacity bottles. The septum vials **560** are held in removable 8-bottle racks to facilitate easy setup and cleaning. The priming volume for each line is about 350 μl. The minimum delivery volume is about 2 μl, and flow rate accuracy is ±5%. The actual amount of material delivered depends on a timed flow of liquid. The flow rate for a particular solvent will

depend on its viscosity and wetting characteristics of the teflon tubing. The flow rate (typically 200-350 μl per sec) is experimentally determined, and this information is contained in the reagent table setup file.

Heating and cooling of the reaction block **526** is effected utilizing a recirculating heat exchanger plate **524**, similar to that found in PCR thermocyclers, that nests with the polypropylene synthesis plate **556** to provide good thermal contact. The liquid contents in a well can be heated or cooled at about 10°C per minute over a range of +5 to +80°C, as polypropylene begins to soften and deform at about 80°C. For temperatures greater than this, a non-disposable synthesis plate machined from stainless steel or monel with replaceable frits can be utilized.

The hardware controller can be any of a wide variety, but conveniently can be designed around a set of three 1 MHz 86332 chips. This controller is used to drive the single X-axis and 8 Y-axis stepper motors as well as provide the timing functions for a total of 154 solenoid valves. Each chip has 16 bidirectional timer I/O and 8 interrupt channels in its timer processing unit (TPU). These are used to provide the step and direction signals, and to read 3 encoder inputs and 2 limit switches for controlling up to three motors per chip. Each 86332 chip also drives a serial chain of 8 UNC5891A darlington array chips to provide power to 64 valves with msec resolution. The controller communicates with the Windows software interface program running on a PC via a 19200 Hz serial channel, and uses an elementary instruction set to communicate valve_number, time_open, motor_number and position_data.

The three components of the software program that run the array synthesizer are the generalized procedure or command (.cmd) file which specifies the synthesis instructions to be performed, the sequence (.seq) file which specifies the scale of the reaction and the order in which variable groups will be added to the core synthon, and the reagent table (.tab) file which specifies the name of a chemical, its location (bottle number), flow rate, and concentration are utilized in conjunction with a basic set of command instructions.

One basic set of command instructions can be:

ADD

IF {block of instructions} END_IF

REPEAT {block of instructions} END_REPEAT

PRIME, NOZZLE_WASH

WAIT, DRAIN

LOAD, REMOVE

NEXT_SEQUENCE

LOOP_BEGIN, LOOP_END

The ADD instruction has two forms, and is intended to have the look and feel of a standard chemical equation. Reagents are specified to be added by a molar amount if the number proceeds the name identifier, or by an absolute volume in microliters if the number follows the identifier. The number of reagents to be added is a parsed list, separated by the "+" sign. For variable reagent identifiers, the key word, <seq>, means look in the sequence table for the identity of the reagent to be added, while the key word, <act>, means add the reagent which is associated with that particular <seq>. Reagents are delivered in the order specified in the list.

Thus:

ADD ACN 300

means: Add 300 μl of the named reagent acetonitrile; ACN to each well of active synthesis

ADD <seq> 300

means: If the sequence pointer in the .seq file is to a reagent in the list of reagents, independent of scale, add 300 μl of that particular reagent specified for that well.

ADD 1.1 PYR + 1.0 <seq> + 1.1 <act1>

means: If the sequence pointer in the .seq file is to a reagent in the list of acids in the Class ACIDS_1, and PYR is the name of pyridine, and ethyl chloroformate is defined in the .tab file to activate the class, ACIDS_1, then this instruction means:

Add 1.1 equiv. pyridine

1.0 equiv. of the acid specified for that well and

1.1 equiv. of the activator, ethyl chloroformate

The IF command allows one to test what type of reagent is specified in the <seq> variable

and process the succeeding block of commands accordingly.

Thus:

```
ACYLATION          {the procedure name}
    BEGIN
        IF CLASS = ACIDS_1
            ADD 1.0 <seq> + 1.1 <act1> + 1.1 PYR
            WAIT 60
        ENDIF
        IF CLASS = ACIDS_2
            ADD 1.0 <seq> + 1.2 <act1> + 1.2 TEA
        ENDIF
        WAIT 60
        DRAIN 10
    END
```

means: Operate on those wells for which reagents contained in the Acid_1 class are specified, WAIT 60 sec, then operate on those wells for which reagents contained in the Acid_2 class are specified, then WAIT 60 sec longer, then DRAIN the whole plate. Note that the Acid_1 group has reacted for a total of 120 sec, while the Acid_2 group has reacted for only 60 sec.

The REPEAT command is a simple way to execute the same block of commands multiple times.

Thus:

```
WASH_1             {the procedure name}
    BEGIN
        REPEAT 3
            ADD ACN 300
            DRAIN 15
        END_REPEAT
    END
```

means: repeats the add acetonitrile and drain sequence for each well three times.

The PRIME command will operate either on specific named reagents or on nozzles

which will be used in the next associated <seq> operation. The μl amount dispensed into a prime port is a constant that can be specified in a config.dat file.

The NOZZLE_WASH command for washing the outside of reaction nozzles free from residue due to evaporation of reagent solvent will operate either on specific named reagents or on nozzles which have been used in the preceding associated <seq> operation. The machine is plumbed such that if any nozzle in a block has been used, all the nozzles in that block will be washed into the prime port.

The WAIT and DRAIN commands are by seconds, with the drain command applying a gas pressure over the top surface of the plate in order to drain the wells.

The LOAD and REMOVE commands are instructions for the machine to pause for operator action.

The NEXT_SEQUENCE command increments the sequence pointer to the next group of substituents to be added in the sequence file. The general form of a .seq file entry is the definition:

Well_No Well_ID Scale Sequence

The sequence information is conveyed by a series of columns, each of which represents a variable reagent to be added at a particular position. The scale (μmole) variable is included so that reactions of different scale can be run at the same time if desired. The reagents are defined in a lookup table (the .tab file), which specifies the name of the reagent as referred to in the sequence and command files, its location (bottle number), flow rate, and concentration. This information is then used by the controller software and hardware to determine both the appropriate slider motion to position the plate and slider arms for delivery of a specific reagent, as well as the specific valve and time required to deliver the appropriate reagents. The adept classification of reagents allows the use of conditional IF loops from within a command file to perform addition of different reagents differently during a "single step" performed across 96 wells simultaneously. The special class ACTIVATORS defines certain reagents that always get added with a particular class of reagents (for example tetrazole during a phosphitylation reaction in adding the next nucleotide to a growing oligonucleotide).

The general form of the .tab file is the definition:

Class	Bottle	Reagent Name	Flow_rate	Conc.

The LOOP_BEGIN and LOOP_END commands define the block of commands which will continue to operate until a NEXT_SEQUENCE command points past the end of the longest list of reactants in any well.

Not included in the command set is a MOVE command. For all of the above commands, if any plate or nozzle movement is required, this is automatically executed in order to perform the desired solvent or reagent delivery operation. This is accomplished by the controller software and hardware, which determines the correct nozzle(s) and well(s) required for a particular reagent addition, then synchronizes the position of the requisite nozzle and well prior to adding the reagent.

A MANUAL mode can also be utilized in which the synthesis plate and nozzle blocks can be "homed" or moved to any position by the operator, the nozzles primed or washed, the various reagent bottles depressurized or washed with solvent, the chamber pressurized, etc. The automatic COMMAND mode can be interrupted at any point, MANUAL commands executed, and then operation resumed at the appropriate location. The sequence pointer can be incremented to restart a synthesis anywhere within a command file.

In reference to Figure 14, the list of oligonucleotides for synthesis can be rearranged or grouped for optimization of synthesis. Thus at process step **574**, the oligonucleotides are grouped according to a factor on which to base the optimization of synthesis. As illustrated in the Examples below, one such factor is the 3' most nucleoside of the oligonucleotide. Using the amidite approach for oligonucleotide synthesis, a nucleotide bearing a 3' phosphoramite is added to the 5' hydroxyl group of a growing nucleotide chain. The first nucleotide (at the 3' terminus of the oligonucleotide - the 3' most nucleoside) is first connected to a solid support. This is normally done batchwise on a large scale as is standard practice during oligonucleotide synthesis.

Such solid supports pre-loaded with a nucleoside are commercially available. In utilizing the multi well format for oligonucleotide synthesis, for each oligonucleotide to be

synthesized, an aliquot of a solid support bearing the proper nucleoside thereon is added to the well for synthesis. Prior to loading the sequence of oligonucleotides to be synthesized in the .seq file, they are sorted by the 3' terminal nucleotide. Based on that sorting, all of the oligonucleotide sequences having an "A" nucleoside at their 3' end are grouped together, those with a "C" nucleoside are grouped together as are those with "G" or "T" nucleosides. Thus in loading the nucleoside-bearing solid support into the synthesis wells, machine movements are conserved.

The oligonucleotides can be grouped by the above described parameter or other parameters that facilitate the synthesis of the oligonucleotides. Thus in Figure 14, sorting is noted as being effected by some parameter of type 1, as for instance the above described 3' most nucleoside, or other types of parameters from type 2 to type n at process steps **576**, **578** and **580**. Since synthesis will be from the 3' end of the oligonucleotides to the 5' end, the oligonucleotide sequences are reverse sorted to read 3' to 5'. The oligonucleotides are entered in the .seq file in this form, i.e., reading 3' to 5'.

Once sorted into types, the position of the oligonucleotides on the synthesis plates is specified at process step **582** by the creation of a .seq file as described above. The .seq file is associated with the respective .cmd and .tab files needed for synthesis of the particular chemistries specified for the oligonucleotides at process step **584** by retrieval of the .cmd and .tab files at process step **586** from the sample database **520**. These files are then input into the multi well synthesizer at process step **588** for oligonucleotide synthesis. Once physically synthesized, the list of oligonucleotides again enters the general procedure flow as indicated in Figure 1. For shipping, storage or other handling purposes, the plates can be lyophilized at this point if desired. Upon lyophilization, each well contains the oligonucleotides located therein as a dry compound.

14. Quality Control.

In an optional step, quality control is performed on the oligonucleotides at process step **600** after a decision is made (decision step **550**) to perform quality control. Although optional, quality control may be desired when there is some reason to think that some aspect of the synthetic process step **500** has been compromised. Alternatively, samples of the oligonucleotides may be taken and stored in the event that the results of assays conducted using the oligonucleotides (process step **700**) yield confusing results or

suboptimal data. In the latter event, for example, quality control might be performed after decision step **800** if no oligonucleotides with sufficient activity are identified. In either event, decision step **650** follows quality control step process **600**. If one or more of the oligonucleotides do not pass quality control, process step **500** can be repeated, i.e., the oligonucleotides are synthesized for a second time.

The operation of the quality control system general procedure **600** is detailed in steps **610-660** of Figure 15. Also referenced in the following discussion are the robotics and associated analytical instrumentation as shown in Figure 18.

During step **610** (Figure 15), sterile, double-distilled water is transferred by an automated liquid handler (**2040** of Figure 18) to each well of a multi-well plate containing a set of lyophilized antisense oligonucleotides. The automated liquid handler (**2040** of Figure 18) reads the barcode sticker on the multi-well plate to obtain the plate's identification number. Automated liquid handler **2040** then queries Sample Database **520** (which resides in Database Server **2002** of Figure 18) for the quality control assay instruction set for that plate and executes the appropriate steps. Three quality control processes are illustrated, however, it is understood that other quality control processes or steps maybe practiced in addition to or in place of the processes illustrated.

The first illustrative quality control process (steps **622** to **626)** quantitates the concentration of oligonucleotide in each well. If this quality control step is performed, an automated liquid handler (**2040** of Figure 18) is instructed to remove an aliquot from each well of the master plate and generate a replicate daughter plate for transfer to the UV spectrophotometer (**2016** of Figure 18). The UV spectrophotometer (**2016** of Figure 18) then measures the optical density of each well at a wavelength of 260 nanometers. Using standardized conversion factors, a microprocessor within UV spectrophotometer (**2016** of Figure 18) then calculates a concentration value from the measured absorbance value for each well and output the results to Sample Database **520**.

The second illustrative quality control process steps **632** to **636)** quantitates the percent of total oligonucleotide in each well that is full length. If this quality control step is performed, an automated liquid handler (**2040** of Figure 18) is instructed to remove an aliquot from each well of the master plate and generate a replicate daughter plate for transfer to the multichannel capillary gel electrophoresis apparatus (**2022** of Figure 18).

The apparatus electrophoretically resolves in capillary tube gels the oligonucleotide product in each well. As the product reaches the distal end of the tube gel during electrophoresis, a detection window dynamically measures the optical density of the product that passes by it. Following electrophoresis, the value of percent product that passed by the detection window with respect to time is utilized by a built in microprocessor to calculate the relative size distribution of oligonucleotide product in each well. These results are then output to the Sample Database (**5**20.

The third illustrative quality control process steps **632** to **636)** quantitates the mass of the oligonucleotide in each well that is full length. If this quality control step is performed, an automated liquid handler (**2040** of Figure 18) is instructed to remove an aliquot from each well of the master plate and generate a replicate daughter plate for transfer to the multichannel liquid electrospray mass spectrometer (**2018** of Figure 18). The apparatus then uses electrospray technology to inject the oligonucleotide product into the mass spectrometer. A built in microprocessor calculates the mass-to-charge ratio to arrive at the mass of oligonucleotide product in each well. The results are then *output to* Sample Database **520**.

Following completion of the selected quality control processes, the output data is manually examined or is examined using an appropriate algorithm and a decision is made as to whether or not the plate receives "Pass" or "Fail" status. The current criteria for acceptance, for 18 mer oligonucleotides, is that at least 85% of the oligonucleotides in a multi-well plate must be 85% or greater full length product as measured by both capillary gel electrophoresis and mass spectrometry. An input (manual or automated) is then made into Sample Database **520** as to the pass/fail status of the plate. If a plate fails, the process cycles back to step **500**, and a new plate of the same oligonucleotides is automatically placed in the plate synthesis request queue (process **554** of Figure 15). If a plate receives "Pass" status, an automated liquid handler (**2040** of Figure 18) is instructed to remove appropriate aliquots from each well of the master plate and generate two replicate daughter plates in which the oligonucleotide in each well is at a concentration of 30 micromolar. The plate then moves on to process **700** for oligonucleotide activity evaluation.

15. Cell Lines for Assaying Oligonucleotide Activity. The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types

provided that the target nucleic acid, or its gene product, is present at measurable levels. This can be routinely determined using, for example, PCR or Northern blot analysis. The following four cell types are provided for illustrative purposes, but other cell types can be routinely used.

T-24 cells: The transitional cell bladder carcinoma cell line T-24 is obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin 100 units per milliliter, and streptomycin 100 micrograms per milliliter (all from Life Technologies). Cells are routinely passaged by trypsinization and dilution when they reach 90% confluence. Cells are routinely seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis. For Northern blotting or other analysis, cells are seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells: The human lung carcinoma cell line A549 is obtained from the ATCC (Manassas, VA). A549 cells were routinely cultured in DMEM basal media (Life Technologies) supplemented with 10% fetal calf serum, penicillin 100 units per milliliter, and streptomycin 100 micrograms per milliliter (all from Life Technologies). Cells are routinely passaged by trypsinization and dilution when they reach 90% confluence.

NHDF cells: Human neonatal dermal fibroblast (NHDF) were obtained from the Clonetics Corporation (Walkersville, MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corp.) as provided by the supplier. Cells are maintained for up to 10 passages as recommended by the supplier.

HEK cells: Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corp. HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corp.) as provided by the supplier. Cell are routinely maintained for up to 10 passages as recommended by the supplier.

16. Treatment of Cells with Candidate Compounds:

When cells reach about 80% confluency, they are treated with oligonucleotide. For cells grown in 96-well plates, wells are washed once with 200 μl OPTI-MEM-1™ reduced-serum medium (Life Technologies) and then treated with 130 μl of OPTI-MEM-

1™ containing 3.75 μg/ml LIPOFECTIN™ (Life Technologies) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16 hours after oligonucleotide treatment.

Alternatively, for cells resistant to cationic mediated transfection, oligonucleotides can be introduced by electroporation. Electroporation conditions must be optimized for every cell type. In general, oligonucleotide is added directly to complete growth media to a final concentration between 1 and 20 micromolar. An electronic pulse is delivered to the cells using a BTX T820 ELECTRO SQUARE PORATOR™ using a Multi-coaxial 96-well electrode (BT840) (BTX Corporation, San Diego, California). Following electroporation, the cells are returned to the incubator for 16 hours.

17. Assaying Oligonucleotide Activity:

Oligonucleotide-mediated modulation of expression of a target nucleic acid can be assayed in a variety of ways known in the art. For example, target RNA levels can be quantitated by, e.g., Northern blot analysis, competitive PCR, or reverse transcriptase polymerase chain reaction (RT-PCR). RNA analysis can be performed on total cellular RNA or, preferably in the case of polypeptide-encoding nucleic acids, poly(A)+ mRNA. For RT-PCR, poly(A)+ mRNA is preferred. Methods of RNA isolation are taught in, for example, Ausubel *et al.* (*Short Protocols in Molecular Biology*, 2nd Ed., pp. 4-1 to 4-13, Greene Publishing Associates and John Wiley & Sons, New York, 1992). Northern blot analysis is routine in the art (*Id.*, pp. 4-14 to 4-29).

Alternatively, total RNA can be prepared from cultured cells or tissue using the QIAGEN RNeasy®-96 kit for the high throughput preparation of RNA (QIAGEN, Inc., Valencia, CA). Essentially, protocols are carried out according to the manufacturer's directions. Optionally, a DNase step is included to remove residual DNA prior to RT-PCR.

To improve efficiency and accuracy the repetitive pipeting steps and elution step have been automated using a QIAGEN Bio-Robot 9604. Essentially after lysing of the oligonucleotide treated cell cultures in situ, the plate is transferred to the robot deck where the pipeting, DNase treatment, and elution steps are carried out.

Reverse transcriptase polymerase chain reaction (RT-PCR) can be conveniently

accomplished using the commercially available ABI PRISM® 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Other methods of PCR are also known in the art.

Target protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), Enzyme-linked immunosorbent assay (ELISA) or fluorescence-activated cell sorting (FACS). Antibodies directed to a protein encoded by a target nucleic acid can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies, (Aerie Corporation, Birmingham, MI or via the internet at http://www.ANTIBODIES-PROBES.com/), or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal, monospecific ("antipeptide") and monoclonal antisera are taught by, for example, Ausubel *et al.* (*Short Protocols in Molecular Biology*, 2nd Ed., pp. 11-3 to 11-54, Greene Publishing Associates and John Wiley & Sons, New York, 1992).

Immunoprecipitation methods are standard in the art and are described by, for example, Ausubel *et al.* (*Id.*, pp. 10-57 to 10-63). Western blot (immunoblot) analysis is standard in the art (*Id.*, pp. 10-32 to 10-10-35). Enzyme-linked immunosorbent assays (ELISA) are standard in the art (*Id.*, pp. 11-5 to 11-17).

Because it is preferred to assay the compounds of the invention in a batchwise fashion, i.e., in parallel to the automated synthesis process described above, preferred means of assaying are suitable for use in 96-well plates and with robotic means. Accordingly, automated RT-PCR is preferred for assaying target nucleic acid levels, and automated ELISA is preferred for assaying target protein levels.

The assaying step, general procedure step **700,** is described in detail in Figure 16. After an appropriate cell line is selected at process step **710**, a decision is made at decision step **714** as to whether RT-PCR will be the only method by which the activity of the compounds is evaluated. In some instances, it is desirable to run alternative assay methods at process step **718**; for example, when it is desired to assess target polypeptide levels as well as target RNA levels, an immunoassay such as an ELISA is run in parallel with the RT-PCR assays. Preferably, such assays are tractable to semi-automated or robotic means.

When RT-PCR is used to evaluate the activities of the compounds, cells are plated into multi-well plates (typically, 96-well plates) in process step **720** and treated with test or

control oligonucleotides in process step **730**. Then, the cells are harvested and lysed in process step **740** and the lysates are introduced into an apparatus where RT-PCR is carried out in process step **750**. A raw data file is generated, and the data is downloaded and compiled at step **760**. Spreadsheet files with data charts are generated at process step **770**, and the experimental data is analyzed at process step **780**. Based on the results, a decision is made at process step **785** as to whether it is necessary to repeat the assays and, if so, the process begins again with step **720**. In any event, data from all the assays on each oligonucleotide are compiled and statistical parameters are automatically determined at process step **790**.

18. Classification of Compounds Based on Their Activity:

Following assaying, general procedure step **700**, oligonucleotide compounds are classified according to one or more desired properties. Typically, three classes of compounds are used: active compounds, marginally active (or "marginal") compounds and inactive compounds. To some degree, the selection criteria for these classes vary from target to target, and members of one or more classes may not be present for a given set of oligonucleotides.

However, some criteria are constant. For example, inactive compounds will typically comprise those compounds having 5% or less inhibition of target expression (relative to basal levels). Active compounds will typically cause at least 30% inhibition of target expression, although lower levels of inhibition are acceptable in some instances. Marginal compounds will have activities intermediate between active and inactive compounds, with preferred marginal compounds having activities more like those of active compounds.

19. Optimization of Lead Compounds by Sequence.

One means by which oligonucleotide compounds are optimized for activity is by varying their nucleobase sequences so that different regions of the target nucleic acid are targeted. Some such regions will be more accessible to oligonucleotide compounds than others, and "sliding" a nucleobase sequence along a target nucleic acid only a few bases can have significant effects on activity. Accordingly, varying or adjusting the nucleobase sequences of the compounds of the invention is one means by which suboptimal compounds can be made optimal, or by which new active compounds can be generated.

The operation of the gene walk process **1100** detailed in steps **1104-1112** of Figure 17 is detailed as follows. As used herein, the term "gene walk" is defined as the process by which a specified oligonucleotide sequence *x* that binds to a specified nucleic acid target *y* is used as a frame of reference around which a series of new oligonucleotides sequences capable of hybridizing to nucleic acid target *y* are generated that are sequence shifted increments of oligonucleotide sequence *x*. Gene walking can be done "downstream", "upstream" or in both directions from a specified oligonucleotide.

During step **1104** the user manually enters the identification number of the oligonucleotide sequence around which it is desired to execute gene walk process **1100** and the name of the corresponding target nucleic acid. The user then enters the scope of the gene walk at step **1104**, by which is meant the number of oligonucleotide sequences that it is desired to generate. The user then enters in step **1108** a positive integer value for the sequence shift increment. Once this data is generated, the gene walk is effected. This causes a subroutine to be executed that automatically generates the desired list of sequences by walking along the target sequence. At that point, the user proceeds to process **400** to assign chemistries to the selected oligonucleotides.

Example 16 below, details a gene walk. In subsequent steps, this new set of nucleobase sequences generated by the gene walk is used to direct the automated synthesis at general procedure step **500** of a second set of candidate oligonucleotides. These compounds are then taken through subsequent process steps to yield active compounds or reiterated as necessary to optimize activity of the compounds.

20. Optimization of Lead Compounds by Chemistry.

Another means by which oligonucleotide compounds of the invention are optimized is by reiterating portions of the process of the invention using marginal or active compounds from the first iteration and selecting additional chemistries to the nucleobase sequences thereof.

Thus, for example, an oligonucleotide chemistry different from that of the first set of oligonucleotides is assigned at general procedure step **400**. The nucleobase sequences of marginal compounds are used to direct the synthesis at general procedure step **500** of a second set of oligonucleotides having the second assigned chemistry. The resulting second set of oligonucleotide compounds is assayed in the same manner as the first set at

procedure process step **700** and the results are examined to determine if compounds having sufficient activity have been generated at decision step **800**.

21. Identification of Sites Amenable to Antisense Technologies.

In a related process, a second oligonucleotide chemistry is assigned at procedure step **400** to the nucleobase sequences of all of the oligonucleotides (or, at least, all of the active and marginal compounds) and a second set of oligonucleotides is synthesized at procedure step **500** having the same nucleobase sequences as the first set of compounds. The resulting second set of oligonucleotide compounds is assayed in the same manner as the first set at procedure step **700** and active and marginal compounds are identified at procedure steps **800** and **1000**.

In order to identify sites on the target nucleic acid that are amenable to a variety of antisense technologies, the following mathematically simple steps are taken. The sequences of active and marginal compounds from two or more such automated syntheses/assays are compared and a set of nucleobase sequences that are active, or marginally so, in both sets of compounds is identified. The reverse complements of these nucleobase sequences corresponds to sequences of the target nucleic acid that are tractable to a variety of antisense and other sequence-based technologies. These antisense-sensitive sites are assembled into contiguous sequences (contigs) using the procedures described for assembling target nucleotide sequences (at procedure step **200**).

22. Systems for Executing Preferred Methods of the Invention.

An embodiment of computer, network and instrument resources for effecting the methods of the invention is shown in Figure 18. In this embodiment, four computer servers are provided. First, a large database server **2002** stores all chemical structure, sample tracking and genomic, assay, quality control, and program status data. Further, this database server serves as the platform for a document management system. Second, a compute engine **2004** runs computational programs including RNA folding, oligonucleotide walking, and genomic searching. Third, a file server **2006** allows raw instrument output storage and sharing of robot instructions. Fourth, a groupware server **2008** enhances staff communication and process scheduling.

A redundant high-speed network system is provided between the main servers and the bridges **2026**, **2028** and **2030**. These bridges provide reliable network access to the

many workstations and instruments deployed for this process. The instruments selected to support this embodiment are all designed to sample directly from standard 96 well microtiter plates, and include an optical density reader **2016**, a combined liquid chromatography and mass spectroscopy instrument **2018**, a gel fluorescence and scintillation imaging system **2032** and **2042**, a capillary gel electrophoreses system **2022** and a real-time PCR system **2034**.

Most liquid handling is accomplished automatically using robots with individually controllable robotic pipetters **2038** and **2020** as well as a 96-well pipette system **2040** for duplicating plates. Windows NT or Macintosh workstations **2044**, **2024**, and **2036** are deployed for instrument control, analysis and productivity support.

23. Relational Database.

Data is stored in an appropriate database. For use with the methods of the invention, a relational database is preferred. Figure 19 illustrates the data structure of a sample relational database. Various elements of data are segregated among linked storage elements of the database.

EXAMPLES

The following examples illustrate the invention and are not intended to limit the same. Those skilled in the art will recognize, or be able to ascertain through routine experimentation, numerous equivalents to the specific procedures, materials and devices described herein. Such equivalents are considered to be within the scope of the present invention.

EXAMPLE 1: Selection of CD40 as a Target

Cell-cell interactions are a feature of a variety of biological processes. In the activation of the immune response, for example, one of the earliest detectable events in a normal inflammatory response is adhesion of leukocytes to the vascular endothelium, followed by migration of leukocytes out of the vasculature to the site of infection or injury. The adhesion of leukocytes to vascular endothelium is an obligate step in their migration out of the vasculature (for a review, see Albelda *et al.*, *FASEB J.*, 1994, *8*, 504). As is well known in the art, cell-cell interactions are also critical for propagation of both B-

lymphocytes and T-lymphocytes resulting in enhanced humoral and cellular immune responses, respectively (for a reviews, see Makgoba *et al.*, *Immunol. Today*, 1989, *10*, 417; Janeway, *Sci. Amer.*, 1993, *269*, 72).

CD40 was first characterized as a receptor expressed on B-lymphocytes. It was later found that engagement of B-cell CD40 with CD40L expressed on activated T-cells is essential for T-cell dependent B-cell activation (i.e. proliferation, immunoglobulin secretion, and class switching) (for a review, see Gruss *et al. Leuk. Lymphoma,* 1997, *24*, 393). A full cDNA sequence for CD40 is available (GenBank accession number X60592, incorporated herein by reference as SEQ ID NO:85).

As interest in CD40 mounted, it was subsequently revealed that functional CD40 is expressed on a variety of cell types other than B-cells, including macrophages, dendritic cells, thymic epithelial cells, Langerhans cells, and endothelial cells (*Ibid.*). These studies have led to the current belief that CD40 plays a much broader role in immune regulation by mediating interactions of T-cells with cell types other than B-cells. In support of this notion, it has been shown that stimulation of CD40 in macrophages and dendritic results is required for T-cell activation during antigen presentation (*Id.*). Recent evidence points to a role for CD40 in tissue inflammation as well. Production of the inflammatory mediators IL-12 and nitric oxide by macrophages has been shown to be CD40 dependent (Buhlmann *et al.*, *J. Clin. Immunol.,* 1996, *16*, 83). In endothelial cells, stimulation of CD40 by CD40L has been found to induce surface expression of E-selectin, ICAM-1, and VCAM-1, promoting adhesion of leukocytes to sites of inflammation (Buhlmann *et al.*, *J. Clin. Immunol,* 1996, *16*, 83; Gruss *et al.*, *Leuk Lymphoma,* 1997, *24*, 393). Finally, a number of reports have documented overexpression of CD40 in epithelial and hematopoietic tumors as well as tumor infiltrating endothelial cells, indicating that CD40 may play a role in tumor growth and/or angiogenesis as well (Gruss *et al.*, *Leuk Lymphoma,* 1997, *24*, 393-422; Kluth *et al. Cancer Res,* 1997, *57*, 891).

Due to the pivotal role that CD40 plays in humoral immunity, the potential exists that therapeutic strategies aimed at downregulating CD40 may provide a novel class of agents useful in treating a number of immune associated disorders, including but not limited to graft versus host disease, graft rejection, and autoimmune diseases such as multiple sclerosis, systemic lupus erythematosus, and certain forms of arthritis. Inhibitors

of CD40 may also prove useful as an anti-inflammatory compound, and could therefore be useful as treatment for a variety of diseases with an inflammatory component such as asthma, rheumatoid arthritis, allograft rejections, inflammatory bowel disease, and various dermatological conditions, including psoriasis. Finally, as more is learned about the association between CD40 overexpression and tumor growth, inhibitors of CD40 may prove useful as anti-tumor agents as well.

Currently, there are no known therapeutic agents which effectively inhibit the synthesis of CD40. To date, strategies aimed at inhibiting CD40 function have involved the use of a variety of agents that disrupt CD40/CD40L binding. These include monoclonal antibodies directed against either CD40 or CD40L, soluble forms of CD40, and synthetic peptides derived from a second CD40 binding protein, A20. The use of neutralizing antibodies against CD40 and/or CD40L in animal models has provided evidence that inhibition of CD40 stimulation would have therapeutic benefit for GVHD, allograft rejection, rheumatoid arthritis, SLE, MS, and B-cell lymphoma (Buhlmann *et al.*, *J. Clin. Immunol,* 1996, *16*, 83). However, due to the expense, short half-life, and bioavailability problems associated with the use of large proteins as therapeutic agents, there is a long felt need for additional agents capable of effectively inhibiting CD40 function. Oligonucleotides compounds avoid many of the pitfalls of current agents used to block CD40/CD40L interactions and may therefore prove to be uniquely useful in a number of therapeutic applications.

EXAMPLE 2: Generation of Virtual Oligonucleotides Targeted to CD40

The process of the invention was used to select oligonucleotides targeted to CD40, generating the list of oligonucleotide sequences with desired properties as shown in Figure 22. From the assembled CD40 sequence, the process began with determining the desired oligonucleotide length to be eighteen nucleotides, as represented in step **2500**. All possible oligonucleotides of this length were generated by Oligo 5.0™, as represented in step **2504**. Desired thermodynamic properties were selected in step **2508**. The single parameter used was oligonucleotides of melting temperature less than or equal to 40°C were discarded. In step **2512**, oligonucleotide melting temperatures were calculated by Oligo 5.0™. Oligonucleotide sequences possessing an undesirable score were discarded. It

is believed that oligonucleotides with melting temperatures near or below physiological and cell culture temperatures will bind poorly to target sequences. All oligonucleotide sequences remaining were exported into a spreadsheet. In step **2516**, desired sequence properties are selected. These include discarding oligonucleotides with at least one stretch of four guanosines in a row and stretches of six of any other nucleotide in a row. In step **2520**, a spreadsheet macro removed all oligonucleotides containing the text string "GGGG." In step **2524**, another spreadsheet macro removed all oligonucleotides containing the text strings "AAAAAA" or "CCCCCC" or "TTTTTT." From the remaining oligonucleotide sequences, 84 sequences were selected manually with the criteria of having an uniform distribution of oligonucleotide sequences throughout the target sequence, as represented in step **2528**. These oligonucleotide sequences were then passed to the next step in the process, assigning actual oligonucleotide chemistries to the sequences.

EXAMPLE 3: Input Files For Automated Oligonucleotide Synthesis Command File (.cmd File)

Table 2 is a command file for synthesis of an oligonucleotide having regions of 2'-O-(2-methoxyethyl) nucleosides and a central region of 2'-deoxy nucleosides each linked by phosphorothioate internucleotide linkages.

Table 2

```
SOLID_SUPPORT_SKIP
        BEGIN
                Next_Sequence
        END

INITIAL-WASH
        BEGIN
                Add ACN 300
                Drain 10
        END
```

```
LOOP-BEGIN
DEBLOCK
        BEGIN
            Prime TCA
            Load Tray
            Repeat 2
                Add TCA 150
                Wait 10
                Drain 8
            End_Repeat
            Remove Tray
                Add TCA 125
                Wait 10
                Drain 8
        END

WASH_AFTER_DEBLOCK
        BEGIN
            Repeat 3
                Add ACN 250 To_All
                Drain 10
            End_Repeat
        END

COUPLING
        BEGIN
            if class = DEOXY_THIOATE
                Nozzle wash <act1>
                prime <act1>
                prime <seq>
                Add <act1> 70 + <seq> 70
```

```
                Wait 40
                        Drain 5
                end-if
        if class = MOE_THIOATE
                Nozzle wash <act1>
                        Prime <act1>
                        prime <seq>
                        Add <act1> 120 + <seq> 120
                        Wait 230
                        Drain 5
                End_if
        END

WASH_AFTER_COUPLING
        BEGIN
                Add ACN 200 To_All
                Drain 10
        END

OXIDIZE
        BEGIN
                if class = DEOXY_THIOATE
        Add BEAU 180
        Wait 40
                        Drain 7
                end_if
                if class = MOE_THIOATE
                Add BEAU 200
                        Wait 120
                        Drain 7
                end_if
```

```
                END

        CAP
                BEGIN
                        Add CAP_B 80 + CAP_A 80
                        Wait 20
                        Drain 7
                END

WASH_AFTER_CAP
                BEGIN
                        Add ACN 150 To_All
                        Drain 5
                        Add ACN 250 To_All
                        Drain 11
                END

        BASE_COUNTER
                BEGIN
                        Next_Sequence
                END

        LOOP_END

        DEBLOCK_FINAL
                BEGIN
                        Prime TCA
                        Load Tray
                        Repeat 2
                Add TCA 150 To_All
                Wait 10
```

```
                Drain 8
                        End_Repeat
                        Remove Tray
                        Add TCA 125 To_All
                        Wait 10
                        Drain 10
                END

        FINAL_WASH
                BEGIN
                        Repeat 4
                                Add ACN 300 to_All
                                Drain_12
                        End_Repeat
                END
ENDALL
                BEGIN
                        Wait 3
                END
```

Sequence files (.seq Files)

Table 3 is a .seq file for oligonucleotides having 2'-deoxy nucleosides linked by phosphorothioate internucleotide linkages.

Table 3

Identity of columns: **Syn #, Well, Scale, Nucleotide at particular position** (identified using base identifier followed by backbone identifier where "s" is phosphorothioate). Note the columns wrap around to next line when longer than one line.

1	A01	200	As	Cs	Cs	As	Gs	Gs	As	Cs	Gs
Gs	Cs	Gs	Gs	As	Cs	Cs	As	G			

	2	A02	200	As	Cs	Gs	Gs	Cs	Gs	Gs	As	Cs
	Cs	As	Gs	As	Gs	Ts	Gs	Gs	A			
	3	A03	200	As	Cs	Cs	As	As	Gs	Cs	As	Gs
	As	Cs	Gs	Gs	As	Gs	As	Cs	G			
5	4	A04	200	As	Gs	Gs	As	Gs	As	Cs	Cs	Cs
	Cs	Gs	As	Cs	Gs	As	As	Cs	G			
	5	A05	200	As	Cs	Cs	Cs	Cs	Gs	As	Cs	Gs
	As	As	Cs	Gs	As	Cs	Ts	Gs	G			
	6	A06	200	As	Cs	Gs	As	As	Cs	Gs	As	Cs
10	Ts	Gs	Gs	Cs	Gs	As	Cs	As	G			
	7	A07	200	As	Cs	Gs	As	Cs	Ts	Gs	Gs	Cs
	Gs	As	Cs	As	Gs	Gs	Ts	As	G			
	8	A08	200	As	Cs	As	Gs	Gs	Ts	As	Gs	Gs
	Ts	Cs	Ts	Ts	Gs	Gs	Ts	Gs	G			
15	9	A09	200	As	Gs	Gs	Ts	Cs	Ts	Ts	Gs	Gs
	Ts	Gs	Gs	Gs	Ts	Gs	As	Cs	G			
	10	A10	200	As	Gs	Ts	Cs	As	Cs	Gs	As	Cs
	As	As	Gs	As	As	As	Cs	As	C			
	11	A11	200	As	Cs	Gs	As	Cs	As	As	Gs	As
20	As	As	Cs	As	Cs	Gs	Gs	Ts	C			
	12	A12	200	As	Gs	As	As	As	Cs	As	Cs	Gs
	Gs	Ts	Cs	Gs	Gs	Ts	Cs	Cs	T			
	13	B01	200	As	As	Cs	As	Cs	Gs	Gs	Ts	Cs
	Gs	Gs	Ts	Cs	Cs	Ts	Gs	Ts	C			
25	14	B02	200	As	Cs	Ts	Cs	As	Cs	Ts	Gs	As
	Cs	Gs	Ts	Gs	Ts	Cs	Ts	Cs	A			
	15	B03	200	As	Cs	Gs	Gs	As	As	Gs	Gs	As
	As	Cs	Gs	Cs	Cs	As	Cs	Ts	T			
	16	B04	200	As	Ts	Cs	Ts	Gs	Ts	Gs	Gs	As
30	Cs	Cs	Ts	Ts	Gs	Ts	Cs	Ts	C			
	17	B05	200	As	Cs	As	Cs	Ts	Ts	Cs	Ts	Ts

	Cs	Cs	Gs	As	Cs	Cs	Gs	Ts	G			
	18	B06	200	As	Cs	Ts	Cs	Ts	Cs	Gs	As	Cs
	As	Cs	As	Gs	Gs	As	Cs	Gs	T			
	19	B07	200	As	As	As	Cs	Cs	Cs	Cs	As	Gs
	Ts	Ts	Cs	Gs	Ts	Cs	Ts	As	A			
	20	B08	200	As	Ts	Gs	Ts	Cs	Cs	Cs	Cs	As
	As	As	Gs	As	Cs	Ts	As	Ts	G			
	21	B09	200	As	Cs	Gs	Cs	Ts	Cs	Gs	Gs	Gs
	As	Cs	Gs	Gs	Gs	Ts	Cs	As	G			
	22	B10	200	As	Gs	Cs	Cs	Gs	As	As	Gs	As
	As	Gs	As	Gs	Gs	Ts	Ts	As	C			
	23	B11	200	As	Cs	As	Cs	As	Gs	Ts	As	Gs
	As	Cs	Gs	As	As	As	Gs	Cs	T			
	24	B12	200	As	Cs	As	Cs	Ts	Cs	Ts	Gs	Gs
	Ts	Ts	Ts	Cs	Ts	Gs	Gs	As	C			
	25	C01	200	As	Cs	Gs	As	Cs	Cs	As	Gs	As
	As	As	Ts	As	Gs	Ts	Ts	Ts	T			
	26	C02	200	As	Gs	Ts	Ts	As	As	As	As	Gs
	Gs	Gs	Cs	Ts	Gs	Cs	Ts	As	G			
	27	C03	200	As	Gs	Gs	Ts	Ts	Gs	Ts	Gs	As
	Cs	Gs	As	Cs	Gs	As	Gs	Gs	T			
	28	C04	200	As	As	Ts	Gs	Ts	As	Cs	Cs	Ts
	As	Cs	Gs	Gs	Ts	Ts	Gs	Gs	C			
	29	C05	200	As	Gs	Ts	Cs	As	Cs	Gs	Ts	Cs
	Cs	Ts	Cs	Ts	Cs	Ts	Gs	Ts	C			
	30	C06	200	Cs	Ts	Gs	Gs	Cs	Gs	As	Cs	As
	Gs	Gs	Ts	As	Gs	Gs	Ts	Cs	T			
	31	C07	200	Cs	Ts	Cs	Ts	Gs	Ts	Gs	Ts	Gs
	As	Cs	Gs	Gs	Ts	Gs	Gs	Ts	C			
	32	C08	200	Cs	As	Gs	Gs	Ts	Cs	Gs	Ts	Cs
	Ts	Ts	Cs	Cs	Cs	Gs	Ts	Gs	G			

	33	C09	200	Cs	Ts	Gs	Ts	Gs	Gs	Ts	As	Gs
	As	Cs	Gs	Ts	Gs	Gs	As	Cs	A			
	34	C10	200	Cs	Ts	As	As	Cs	Gs	As	Ts	Gs
	Ts	Cs	Cs	Cs	Cs	As	As	As	G			
5	35	C11	200	Cs	Ts	Gs	Ts	Ts	Cs	Gs	As	Cs
	As	Cs	Ts	Cs	Ts	Gs	Gs	Ts	T			
	36	C12	200	Cs	Ts	Gs	Gs	As	Cs	Cs	As	As
	Cs	As	Cs	Gs	Ts	Ts	Gs	Ts	C			
	37	D01	200	Cs	Cs	Gs	Ts	Cs	Cs	Gs	Ts	Gs
10	Ts	Ts	Ts	Gs	Ts	Ts	Cs	Ts	G			
	38	D02	200	Cs	Ts	Gs	As	Cs	Ts	As	Cs	As
	As	Cs	As	Gs	As	Cs	As	Cs	C			
	39	D03	200	Cs	As	As	Cs	As	Gs	As	Cs	As
	Cs	Cs	As	Gs	Gs	Gs	Gs	Ts	C			
15	40	D04	200	Cs	As	Gs	Gs	Gs	Gs	Ts	Cs	Cs
	Ts	As	Gs	Cs	Cs	Gs	As	Cs	T			
	41	D05	200	Cs	Ts	Cs	Ts	As	Gs	Ts	Ts	As
	As	As	As	Gs	Gs	Gs	Cs	Ts	G			
	42	D06	200	Cs	Ts	Gs	Cs	Ts	As	Gs	As	As
20	Gs	Gs	As	Cs	Cs	Gs	As	Gs	G			
	43	D07	200	Cs	Ts	Gs	As	As	As	Ts	Gs	Ts
	As	Cs	Cs	Ts	As	Cs	Gs	Gs	T			
	44	D08	200	Cs	As	Cs	Cs	Cs	Gs	Ts	Ts	Ts
	Gs	Ts	Cs	Cs	Gs	Ts	Cs	As	A			
25	45	D09	200	Cs	Ts	Cs	Gs	As	Ts	As	Cs	Gs
	Gs	Gs	Ts	Cs	As	Gs	Ts	Cs	A			
	46	D10	200	Gs	Gs	Ts	As	Gs	Gs	Ts	Cs	Ts
	Ts	Gs	Gs	Ts	Gs	Gs	Gs	Ts	G			
	47	D11	200	Gs	As	Cs	Ts	Ts	Ts	Gs	Cs	Cs
30	Ts	Ts	As	Cs	Gs	Gs	As	As	G			
	48	D12	200	Gs	Ts	Gs	Gs	As	Gs	Ts	Cs	Ts

	Ts	Ts	Gs	Ts	Cs	Ts	Gs	Ts	G			
	49	E01	200	Gs	Gs	As	Gs	Ts	Cs	Ts	Ts	Ts
	Gs	Ts	Cs	Ts	Gs	Ts	Gs	Gs	T			
	50	E02	200	Gs	Gs	As	Cs	As	Cs	Ts	Cs	Ts
	Cs	Gs	As	Cs	As	Cs	As	Gs	G			
	51	E03	200	Gs	As	Cs	As	Cs	As	Gs	Gs	As
	Cs	Gs	Ts	Gs	Gs	Cs	Gs	As	G			
	52	E04	200	Gs	As	Gs	Ts	As	Cs	Gs	As	Gs
	Cs	Gs	Gs	Gs	Cs	Cs	Gs	As	A			
	53	E05	200	Gs	As	Cs	Ts	As	Ts	Gs	Gs	Ts
	As	Gs	As	Cs	Gs	Cs	Ts	Cs	G			
	54	E06	200	Gs	As	As	Gs	As	Gs	Gs	Ts	Ts
	As	Cs	As	Cs	As	Gs	Ts	As	G			
	55	E07	200	Gs	As	Gs	Gs	Ts	Ts	As	Cs	As
	Cs	As	Gs	Ts	As	Gs	As	Cs	G			
	56	E08	200	Gs	Ts	Ts	Gs	Ts	Cs	Cs	Gs	Ts
	Cs	Cs	Gs	Ts	Gs	Ts	Ts	Ts	G			
	57	E09	200	Gs	As	Cs	Ts	Cs	Ts	Cs	Gs	Gs
	Gs	As	Cs	Cs	As	Cs	Cs	As	C			
	58	E10	200	Gs	Ts	As	Gs	Gs	As	Gs	As	As
	Cs	Cs	As	Cs	Gs	As	Cs	Cs	A			
	59	E11	200	Gs	Gs	Ts	Ts	Cs	Ts	Ts	Cs	Gs
	Gs	Ts	Ts	Gs	Gs	Ts	Ts	As	T			
	60	E12	200	Gs	Ts	Gs	Gs	Gs	Gs	Ts	Ts	Cs
	Gs	Ts	Cs	Cs	Ts	Ts	Gs	Gs	G			
	61	F01	200	Gs	Ts	Cs	As	Cs	Gs	Ts	Cs	Cs
	Ts	Cs	Ts	Gs	As	As	As	Ts	G			
	62	F02	200	Gs	Ts	Cs	Cs	Ts	Cs	Cs	Ts	As
	Cs	Cs	Gs	Ts	Ts	Ts	Cs	Ts	C			
	63	F03	200	Gs	Ts	Cs	Cs	Cs	Cs	As	Cs	Gs
	Ts	Cs	Cs	Gs	Ts	Cs	Ts	Ts	C			

	64	F04	200	Ts	Cs	As	Cs	Cs	As	Gs	Gs	As
	Cs	Gs	Gs	Cs	Gs	Gs	As	Cs	C			
	65	F05	200	Ts	As	Cs	Cs	As	As	Gs	Cs	As
	Gs	As	Cs	Gs	Gs	As	Gs	As	C			
5	66	F06	200	Ts	Cs	Cs	Ts	Gs	Ts	Cs	Ts	Ts
	Ts	Gs	As	Cs	Cs	As	Cs	Ts	C			
	67	F07	200	Ts	Gs	Ts	Cs	Ts	Ts	Ts	Gs	As
	Cs	Cs	As	Cs	Ts	Cs	As	Cs	T			
	68	F08	200	Ts	Gs	As	Cs	Cs	As	Cs	Ts	Cs
10	As	Cs	Ts	Gs	As	Cs	Gs	Ts	G			
	69	F09	200	Ts	Gs	As	Cs	Gs	Ts	Gs	Ts	Cs
	Ts	Cs	As	As	Gs	Ts	Gs	As	C			
	70	F10	200	Ts	Cs	As	As	Gs	Ts	Gs	As	Cs
	Ts	Ts	Ts	Gs	Cs	Cs	Ts	Ts	A			
15	71	F11	200	Ts	Gs	Ts	Ts	Ts	As	Ts	Gs	As
	Cs	Gs	Cs	Ts	Gs	Gs	Gs	Gs	T			
	72	F12	200	Ts	Ts	As	Ts	Gs	As	Cs	Gs	Cs
	Ts	Gs	Gs	Gs	Gs	Ts	Ts	Gs	G			
	73	G01	200	Ts	Gs	As	Cs	Gs	Cs	Ts	Gs	Gs
20	Gs	Gs	Ts	Ts	Gs	Gs	As	Ts	C			
	74	G02	200	Ts	Cs	Gs	Ts	Cs	Ts	Ts	Cs	Cs
	Cs	Gs	Ts	Gs	Gs	As	Gs	Ts	C			
	75	G03	200	Ts	Gs	Gs	Ts	As	Gs	As	Cs	Gs
	Ts	Gs	Gs	As	Cs	As	Cs	Ts	T			
25	76	G04	200	Ts	Ts	Cs	Ts	Ts	Cs	Cs	Gs	As
	Cs	Cs	Gs	Ts	Gs	As	Cs	As	T			
	77	G05	200	Ts	Gs	Gs	Ts	As	Gs	As	Cs	Gs
	Cs	Ts	Cs	Gs	Gs	Gs	As	Cs	G			
	78	G06	200	Ts	As	Gs	As	Cs	Gs	Cs	Ts	Cs
30	Gs	Gs	Gs	As	Cs	Gs	Gs	Gs	T			
	79	G07	200	Ts	Ts	Ts	Ts	As	Cs	As	Gs	Ts

	Gs	Gs	Gs	As	As	Cs	Cs	Ts	G			
	80	G08	200	Ts	Gs	Gs	Gs	As	As	Cs	Cs	Ts
	Gs	Ts	Ts	Cs	Gs	As	Cs	As	C			
	81	G09	200	Ts	Cs	Gs	Gs	Gs	As	Cs	Cs	As
	Cs	Cs	As	Cs	Ts	As	Gs	Gs	G			
	82	G10	200	Ts	As	Gs	Gs	As	Cs	As	As	As
	Cs	Gs	Gs	Ts	As	Gs	Gs	As	G			
	83	G11	200	Ts	Gs	Cs	Ts	As	Gs	As	As	Gs
	Gs	As	Cs	Cs	Gs	As	Gs	Gs	T			
	84	G12	200	Ts	Cs	Ts	Gs	Ts	Cs	As	Cs	Ts
	Cs	Cs	Gs	As	Cs	Gs	Ts	Gs	G			

Table 4 is a .seq file for oligonucleotides having regions of 2'-O-(2-methoxyethyl)-nucleosides and a central region of 2'-deoxy nucleosides each linked by phosphorothioate internucleotide linkages.

Table 4

Identity of columns: **Syn #, Well, Scale, Nucleotide at particular position** (identified using base identifier followed by backbone identifier where "s" is phosphorothioate and "moe" indicated a 2'-O-(2-methoxyethyl) substituted nucleoside). The columns wrap around to next line when longer than one line.

1 A01 200 moeAs moeCs moeCs moeAs Gs Gs As Cs Gs Gs Cs Gs Gs As moeCs moeCs moeAs moeG

2 A02 200 moeAs moeCs moeGs moeGs Cs Gs Gs As Cs Cs As Gs As Gs moeTs moeGs moeGs moeA

3 A03 200 moeAs moeCs moeCs moeAs As Gs Cs As Gs As Cs Gs Gs As moeGs moeAs moeCs moeG

4 A04 200 moeAs moeGs moeGs moeAs Gs As Cs Cs Cs Cs Gs As Cs Gs moeAs moeAs moeCs moeG

5 A05 200 moeAs moeCs moeCs moeCs Cs Gs As Cs Gs As As Cs Gs As

moeCs moeTs moeGs moeG

6 A06 200 moeAs moeCs moeGs moeAs As Cs Gs As Cs Ts Gs Gs Cs Gs moeAs moeCs moeAs moeG

7 A07 200 moeAs moeCs moeGs moeAs Cs Ts Gs Gs Cs Gs As Cs As Gs moeGs moeTs moeAs moeG

8 A08 200 moeAs moeCs moeAs moeGs Gs Ts As Gs Gs Ts Cs Ts Ts Gs moeGs moeTs moeGs moeG

9 A09 200 moeAs moeGs moeGs moeTs Cs Ts Ts Gs Gs Ts Gs Gs Gs Ts moeGs moeAs moeCs moeG

10 A10 200 moeAs moeGs moeTs moeCs As Cs Gs As Cs As As Gs As As moeAs moeCs moeAs moeC

11 A11 200 moeAs moeCs moeGs moeAs Cs As As Gs As As As Cs As Cs moeGs moeGs moeTs moeC

12 A12 200 moeAs moeGs moeAs moeAs As Cs As Cs Gs Gs Ts Cs Gs Gs moeTs moeCs moeCs moeT

13 B01 200 moeAs moeAs moeCs moeAs Cs Gs Gs Ts Cs Gs Gs Ts Cs Cs moeTs moeGs moeTs moeC

14 B02 200 moeAs moeCs moeTs moeCs As Cs Ts Gs As Cs Gs Ts Gs Ts moeCs moeTs moeCs moeA

15 B03 200 moeAs moeCs moeGs moeGs As As Gs Gs As As Cs Gs Cs Cs moeAs moeCs moeTs moeT

16 B04 200 moeAs moeTs moeCs moeTs Gs Ts Gs Gs As Cs Cs Ts Ts Gs moeTs moeCs moeTs moeC

17 B05 200 moeAs moeCs moeAs moeCs Ts Ts Cs Ts Ts Cs Cs Gs As Cs moeCs moeGs moeTs moeG

18 B06 200 moeAs moeCs moeTs moeCs Ts Cs Gs As Cs As Cs As Gs Gs moeAs moeCs moeGs moeT

19 B07 200 moeAs moeAs moeAs moeCs Cs Cs Cs As Gs Ts Ts Cs Gs Ts moeCs moeTs moeAs moeA

20 B08 200 moeAs moeTs moeGs moeTs Cs Cs Cs Cs As As As Gs As Cs moeTs moeAs moeTs moeG

21 B09 200 moeAs moeCs moeGs moeCs Ts Cs Gs Gs Gs As Cs Gs Gs Gs moeTs moeCs moeAs moeG

22 B10 200 moeAs moeGs moeCs moeCs Gs As As Gs As As Gs As Gs Gs moeTs moeTs moeAs moeC

23 B11 200 moeAs moeCs moeAs moeCs As Gs Ts As Gs As Cs Gs As As moeAs moeGs moeCs moeT

24 B12 200 moeAs moeCs moeAs moeCs Ts Cs Ts Gs Gs Ts Ts Ts Cs Ts moeGs moeGs moeAs moeC

25 C01 200 moeAs moeCs moeGs moeAs Cs Cs As Gs As As As Ts As Gs moeTs moeTs moeTs moeT

26 C02 200 moeAs moeGs moeTs moeTs As As As As Gs Gs Gs Cs Ts Gs moeCs moeTs moeAs moeG

27 C03 200 moeAs moeGs moeGs moeTs Ts Gs Ts Gs As Cs Gs As Cs Gs moeAs moeGs moeGs moeT

28 C04 200 moeAs moeAs moeTs moeGs Ts As Cs Cs Ts As Cs Gs Gs Ts moeTs moeGs moeGs moeC

29 C05 200 moeAs moeGs moeTs moeCs As Cs Gs Ts Cs Cs Ts Cs Ts Cs moeTs moeGs moeTs moeC

30 C06 200 moeCs moeTs moeGs moeGs Cs Gs As Cs As Gs Gs Ts As Gs moeGs moeTs moeCs moeT

31 C07 200 moeCs moeTs moeCs moeTs Gs Ts Gs Ts Gs As Cs Gs Gs Ts moeGs moeGs moeTs moeC

32 C08 200 moeCs moeAs moeGs moeGs Ts Cs Gs Ts Cs Ts Ts Cs Cs Cs moeGs moeTs moeGs moeG

33 C09 200 moeCs moeTs moeGs moeTs Gs Gs Ts As Gs As Cs Gs Ts Gs moeGs moeAs moeCs moeA

34 C10 200 moeCs moeTs moeAs moeAs Cs Gs As Ts Gs Ts Cs Cs Cs Cs moeAs moeAs moeAs moeG

35 C11 200 moeCs moeTs moeGs moeTs Ts Cs Gs As Cs As Cs Ts Cs Ts moeGs moeGs moeTs moeT

36 C12 200 moeCs moeTs moeGs moeGs As Cs Cs As As Cs As Cs Gs Ts

moeTs moeGs moeTs moeC

37 D01 200 moeCs moeCs moeGs moeTs Cs Cs Gs Ts Gs Ts Ts Ts Gs Ts moeTs moeCs moeTs moeG

38 D02 200 moeCs moeTs moeGs moeAs Cs Ts As Cs As As Cs As Gs As moeCs moeAs moeCs moeC

39 D03 200 moeCs moeAs moeAs moeCs As Gs As Cs As Cs Cs As Gs Gs moeGs moeGs moeTs moeC

40 D04 200 moeCs moeAs moeGs moeGs Gs Gs Ts Cs Cs Ts As Gs Cs Cs moeGs moeAs moeCs moeT

41 D05 200 moeCs moeTs moeCs moeTs As Gs Ts Ts As As As As Gs Gs moeGs moeCs moeTs moeG

42 D06 200 moeCs moeTs moeGs moeCs Ts As Gs As As Gs Gs As Cs Cs moeGs moeAs moeGs moeG

43 D07 200 moeCs moeTs moeGs moeAs As As Ts Gs Ts As Cs Cs Ts As moeCs moeGs moeGs moeT

44 D08 200 moeCs moeAs moeCs moeCs Cs Gs Ts Ts Ts Gs Ts Cs Cs Gs moeTs moeCs moeAs moeA

45 D09 200 moeCs moeTs moeCs moeGs As Ts As Cs Gs Gs Gs Ts Cs As moeGs moeTs moeCs moeA

46 D10 200 moeGs moeGs moeTs moeAs Gs Gs Ts Cs Ts Ts Gs Gs Ts Gs moeGs moeGs moeTs moeG

47 D11 200 moeGs moeAs moeCs moeTs Ts Ts Gs Cs Cs Ts Ts As Cs Gs moeGs moeAs moeAs moeG

48 D12 200 moeGs moeTs moeGs moeGs As Gs Ts Cs Ts Ts Ts Gs Ts Cs moeTs moeGs moeTs moeG

49 E01 200 moeGs moeGs moeAs moeGs Ts Cs Ts Ts Ts Gs Ts Cs Ts Gs moeTs moeGs moeGs moeT

50 E02 200 moeGs moeGs moeAs moeCs As Cs Ts Cs Ts Cs Gs As Cs As moeCs moeAs moeGs moeG

51 E03 200 moeGs moeAs moeCs moeAs Cs As Gs Gs As Cs Gs Ts Gs Gs moeCs moeGs moeAs moeG

52 E04 200 moeGs moeAs moeGs moeTs As Cs Gs As Gs Cs Gs Gs Gs Cs moeCs moeGs moeAs moeA

53 E05 200 moeGs moeAs moeCs moeTs As Ts Gs Gs Ts As Gs As Cs Gs moeCs moeTs moeCs moeG

54 E06 200 moeGs moeAs moeAs moeGs As Gs Gs Ts Ts As Cs As Cs As moeGs moeTs moeAs moeG

55 E07 200 moeGs moeAs moeGs moeGs Ts Ts As Cs As Cs As Gs Ts As moeGs moeAs moeCs moeG

56 E08 200 moeGs moeTs moeTs moeGs Ts Cs Cs Gs Ts Cs Cs Gs Ts Gs moeTs moeTs moeTs moeG

57 E09 200 moeGs moeAs moeCs moeTs Cs Ts Cs Gs Gs Gs As Cs Cs As moeCs moeCs moeAs moeC

58 E10 200 moeGs moeTs moeAs moeGs Gs As Gs As As Cs Cs As Cs Gs moeAs moeCs moeCs moeA

59 E11 200 moeGs moeGs moeTs moeTs Cs Ts Ts Cs Gs Gs Ts Ts Gs Gs moeTs moeTs moeAs moeT

60 E12 200 moeGs moeTs moeGs moeGs Gs Gs Ts Ts Cs Gs Ts Cs Cs Ts moeTs moeGs moeGs moeG

61 F01 200 moeGs moeTs moeCs moeAs Cs Gs Ts Cs Cs Ts Cs Ts Gs As moeAs moeAs moeTs moeG

62 F02 200 moeGs moeTs moeCs moeCs Ts Cs Cs Ts As Cs Cs Gs Ts Ts moeTs moeCs moeTs moeC

63 F03 200 moeGs moeTs moeCs moeCs Cs Cs As Cs Gs Ts Cs Cs Gs Ts moeCs moeTs moeTs moeC

64 F04 200 moeTs moeCs moeAs moeCs Cs As Gs Gs As Cs Gs Gs Cs Gs moeGs moeAs moeCs moeC

65 F05 200 moeTs moeAs moeCs moeCs As As Gs Cs As Gs As Cs Gs Gs moeAs moeGs moeAs moeC

66 F06 200 moeTs moeCs moeCs moeTs Gs Ts Cs Ts Ts Ts Gs As Cs Cs moeAs moeCs moeTs moeC

67 F07 200 moeTs moeGs moeTs moeCs Ts Ts Ts Gs As Cs Cs As Cs Ts

moeCs moeAs moeCs moeT

68 F08 200 moeTs moeGs moeAs moeCs Cs As Cs Ts Cs As Cs Ts Gs As moeCs moeGs moeTs moeG

69 F09 200 moeTs moeGs moeAs moeCs Gs Ts Gs Ts Cs Ts Cs As As Gs moeTs moeGs moeAs moeC

70 F10 200 moeTs moeCs moeAs moeAs Gs Ts Gs As Cs Ts Ts Ts Gs Cs moeCs moeTs moeTs moeA

71 F11 200 moeTs moeGs moeTs moeTs Ts As Ts Gs As Cs Gs Cs Ts Gs moeGs moeGs moeGs moeT

72 F12 200 moeTs moeTs moeAs moeTs Gs As Cs Gs Cs Ts Gs Gs Gs Gs moeTs moeTs moeGs moeG

73 G01 200 moeTs moeGs moeAs moeCs Gs Cs Ts Gs Gs Gs Gs Ts Ts Gs moeGs moeAs moeTs moeC

74 G02 200 moeTs moeCs moeGs moeTs Cs Ts Ts Cs Cs Cs Gs Ts Gs Gs moeAs moeGs moeTs moeC

75 G03 200 moeTs moeGs moeGs moeTs As Gs As Cs Gs Ts Gs Gs As Cs moeAs moeCs moeTs moeT

76 G04 200 moeTs moeTs moeCs moeTs Ts Cs Cs Gs As Cs Cs Gs Ts Gs moeAs moeCs moeAs moeT

77 G05 200 moeTs moeGs moeGs moeTs As Gs As Cs Gs Cs Ts Cs Gs Gs moeGs moeAs moeCs moeG

78 G06 200 moeTs moeAs moeGs moeAs Cs Gs Cs Ts Cs Gs Gs Gs As Cs moeGs moeGs moeGs moeT

79 G07 200 moeTs moeTs moeTs moeTs As Cs As Gs Ts Gs Gs Gs As As moeCs moeCs moeTs moeG

80 G08 200 moeTs moeGs moeGs moeGs As As Cs Cs Ts Gs Ts Ts Cs Gs moeAs moeCs moeAs moeC

81 G09 200 moeTs moeCs moeGs moeGs Gs As Cs Cs As Cs Cs As Cs Ts moeAs moeGs moeGs moeG

82 G10 200 moeTs moeAs moeGs moeGs As Cs As As As Cs Gs Gs Ts As moeGs moeGs moeAs moeG

83 G11 200 moeTs moeGs moeCs moeTs As Gs As As Gs Gs As Cs Cs Gs moeAs moeGs moeGs moeT

84 G12 200 moeTs moeCs moeTs moeGs Ts Cs As Cs Ts Cs Cs Gs As Cs moeGs moeTs moeGs moeG

Reagent file (.tab File)

Table 5 is a .tab file for reagents necessary for synthesizing an oligonucleotides having both 2'-O-(2-methoxyethyl)nucleosides and 2'-deoxy nucleosides located therein.

Table 5

Identity of columns: **GroupName, Bottle ID, ReagentName, FlowRate, Concentration.** Wherein reagent name is identified using base identifier, "moe" indicated a 2'-O-(2-methoxyethyl) substituted nucleoside and "cpg" indicates a control pore glass solid support medium. The columns wrap around to next line when longer than one line.

SUPPORT

BEGIN

0	moeG	moeG	cpg	100	1
0	moe5meC	moe5meC	cpg	100	1
0	moeA	moeA	cpg	100	1
0	moeT	moeT	cpg	100	1

END

DEBLOCK

BEGIN

70	TCA	TCA	100	1

END

WASH

BEGIN

65	ACN	ACN	190	1

END

OXIDIZERS

BEGIN

68	BEAU	BEAUCAGE	320	1	

END

CAPPING

BEGIN

66	CAP_B	CAP_B	220	1
67	CAP_A	CAP_A	230	1

END

DEOXY THIOATE

BEGIN

31,32	Gs	deoxyG	270	1
39,40	5meCs	5methyldeoxyC	270	1
37,38	As	deoxyA	270	1
29,30	Ts	deoxyT	270	1

END

MOE-THIOATE

BEGIN

15,16	moeGs	methoxyethoxyG	240	1
23,24	moe5meCs	methoxyethoxyC	240	1
21,22	moeAs	methoxyethoxyA	240	1
13,14	moeTs	methoxyethoxyT	240	1

END

ACTIVATORS

BEGIN

5,6,7,8	SET	s-ethyl-tet	280

Activates

DEOXY_THIOATE

MOE_THIOATE

END

EXAMPLE 4: Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry using a multi well automated synthesizer utilizing input files as described in EXAMPLE 3 above. The oligonucleotides were synthesized by assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyl-diisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE/ABI, Pharmacia). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Following synthesis, oligonucleotides were cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried *in vacuo*. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

EXAMPLE 5: Alternative Oligonucleotide Synthesis

Unsubstituted and substituted phosphodiester oligonucleotides are alternately synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates are synthesized as per the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping

step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, incorporated herein by reference in its entirety.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, incorporated herein by reference in its entirety.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, each of which is incorporated herein by reference in its entirety.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, incorporated herein by reference in its entirety.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), each of which is incorporated herein by reference in its entirety.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, incorporated herein by reference in its entirety.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, incorporated herein by reference in its entirety.

Boranophosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, each of which is incorporated herein by reference in its entirety.

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and PO or PS linkages are prepared as described in U.S. Patents 5,378,825; 5,386,023; 5,489,677; 5,602,240 and 5,610,289, each of which is incorporated herein by reference in its entirety.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in

U.S. Patents 5,264,562 and 5,264,564, each of which is incorporated herein by reference in its entirety.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, incorporated herein by reference in its entirety.

EXAMPLE 6: PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry,* 1996, *4,* 5. They may also be prepared in accordance with U.S. Patents 5,539,082; 5,700,922, and 5,719,262, each of which is incorporated herein by reference in its entirety.

EXAMPLE 7: Chimeric Oligonucleotide Synthesis

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers."

A. [2'-O-Me]--[2'-deoxy]--[2'-O-Me] Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidites for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidites for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for DNA and twice for 2'-0-methyl. The fully protected oligonucleotide was cleaved from the support and the phosphate group is deprotected in 3:1 Ammonia/Ethanol at room temperature overnight then lyophilized to dryness.

Treatment in methanolic ammonia for 24 hrs at room temperature is done to deprotect all bases and the samples are again lyophilized to dryness.

B. [2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(2-Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(2-methoxyethyl)] chimeric phosphorothioate oligonucleotides are prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(2-methoxyethyl) amidites for the 2'-O-methyl amidites.

C. [2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotide

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(2-methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(2-methoxyethyl) amidites for the 2'-O-methyl amidites in the wing portions. Sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) is used to generate the phosphorothioate internucleotide linkages within the wing portions of the chimeric structures. Oxidization with iodine is used to generate the phosphodiester internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States Patent 5,623,065, which is incorporated herein by reference in its entirety.

EXAMPLE 8: Output Oligonucleotides From Automated Oligonucleotide Synthesis

Using the .seq files, the .cmd files and .tab file of Example 3, oligonucleotides were prepared as per the protocol of the 96 well format of Example 4. The oligonucleotides were prepared utilizing phosphorothioate chemistry to give in one instance a first library of phosphorothioate oligodeoxynucleotides. The oligonucleotides were prepared in a second instance as a second library of hybrid oligonucleotides having phosphorothioate backbones with a first and third "wing" region of 2'-O-(2-methoxyethyl)nucleotides on either side of a center gap region of 2'-deoxy nucleotides. The two libraries contained the same set of oligonucleotide sequences. Thus the two

libraries are redundant with respect to sequence but are unique with respect to the combination of sequence and chemistry. Because the sequences of the second library of compounds is the same as the first (however the chemistry is different), for brevity sake, the second library is not shown.

For illustrative purposes Tables 6-a and 6-b show the sequences of an initial first library, i.e., a library of phosphorothioate oligonucleotides targeted to a CD40 target. The compounds of Table 6-a shows the members of this library listed in compliance with the established rule for listing SEQ ID NO:, i.e., in numerical SEQ ID NO: order.

Table 6-a

Sequences of Oligonucleotides Targeted to CD40 by SEQ ID NO.:

NUCLEOBASE SEQUENCE	SEQ ID NO.
CCAGGCGGCAGGACCACT	1
GACCAGGCGGCAGGACCA	2
AGGTGAGACCAGGCGGCA	3
CAGAGGCAGACGAACCAT	4
GCAGAGGCAGACGAACCA	5
GCAAGCAGCCCCAGAGGA	6
GGTCAGCAAGCAGCCCCA	7
GACAGCGGTCAGCAAGCA	8
GATGGACAGCGGTCAGCA	9
TCTGGATGGACAGCGGTC	10
GGTGGTTCTGGATGGACA	11
GTGGGTGGTTCTGGATGG	12
GCAGTGGGTGGTTCTGGA	13
CACAAAGAACAGCACTGA	14
CTGGCACAAAGAACAGCA	15
TCCTGGCTGGCACAAAGA	16
CTGTCCTGGCTGGCACAA	17
CTCACCAGTTTCTGTCCT	18
TCACTCACCAGTTTCTGT	19

	GTGCAGTCACTCACCAGT	20
	ACTCTGTGCAGTCACTCA	21
	CAGTGAACTCTGTGCAGT	22
	ATTCCGTTTCAGTGAACT	23
5	GAAGGCATTCCGTTTCAG	24
	TTCACCGCAAGGAAGGCA	25
	CTCTGTTCCAGGTGTCTA	26
	CTGGTGGCAGTGTGTCTC	27
	TGGGGTCGCAGTATTTGT	28
10	GGTTGGGGTCGCAGTATT	29
	CTAGGTTGGGGTCGCAGT	30
	GGTGCCCTTCTGCTGGAC	31
	CTGAGGTGCCCTTCTGCT	32
	GTGTCTGTTTCTGAGGTG	33
15	TGGTGTCTGTTTCTGAGG	34
	ACAGGTGCAGATGGTGTC	35
	TTCACAGGTGCAGATGGT	36
	GTGCCAGCCTTCTTCACA	37
	TACAGTGCCAGCCTTCTT	38
20	GGACACAGCTCTCACAGG	39
	TGCAGGACACAGCTCTCA	40
	GAGCGGTGCAGGACACAG	41
	AAGCCGGGCGAGCATGAG	42
	AATCTGCTTGACCCCAAA	43
25	GAAACCCCTGTAGCAATC	44
	GTATCAGAAACCCCTGTA	45
	GCTCGCAGATGGTATCAG	46
	GCAGGGCTCGCAGATGGT	47
	TGGGCAGGGCTCGCAGAT	48
30	GACTGGGCAGGGCTCGCA	49
	CATTGGAGAAGAAGCCGA	50

	GATGACACATTGGAGAAG	51
	GCAGATGACACATTGGAG	52
	TCGAAAGCAGATGACACA	53
	GTCCAAGGGTGACATTTT	54
5	CACAGCTTGTCCAAGGGT	55
	TTGGTCTCACAGCTTGTC	56
	CAGGTCTTTGGTCTCACA	57
	CTGTTGCACAACCAGGTC	58
	GTTTGTGCCTGCCTGTTG	59
10	GTCTTGTTTGTGCCTGCC	60
	CCACAGACAACATCAGTC	61
	CTGGGGACCACAGACAAC	62
	TCAGCCGATCCTGGGGAC	63
	CACCACCAGGGCTCTCAG	64
15	GGGATCACCACCAGGGCT	65
	GAGGATGGCAAACAGGAT	66
	ACCAGCACCAAGAGGATG	67
	TTTTGATAAAGACCAGCA	68
	TATTGGTTGGCTTCTTGG	69
20	GGGTTCCTGCTTGGGGTG	70
	GTCGGGAAAATTGATCTC	71
	GATCGTCGGGAAAATTGA	72
	GGAGCCAGGAAGATCGTC	73
	TGGAGCCAGGAAGATCGT	74
25	TGGAGCAGCAGTGTTGGA	75
	GTAAAGTCTCCTGCACTG	76
	TGGCATCCATGTAAAGTC	77
	CGGTTGGCATCCATGTAA	78
	CTCTTTGCCATCCTCCTG	79
30	CTGTCTCTCCTGCACTGA	80
	GGTGCAGCCTCACTGTCT	81

AACTGCCTGTTTGCCCAC	82
CTTCTGCCTGCACCCCTG	83
ACTGACTGGGCATAGCTC	84

The sequences shown in Table 6-a, above, and Table 6-b, below, are in a 5' to 3' direction. This is reversed with respect to 3' to 5' direction shown in the .seq files of Example 3. For synthesis purposes, the .seq files are generated reading from 3' to 5'. This allows for aligning all of the 3' most "A" nucleosides together, all of the 3' most "G" nucleosides together, all of the 3' most "C" nucleosides together and all of the 3' most "T" nucleosides together. Thus when the first nucleoside of each particular oligonucleotide (attached to the solid support) is added to the wells on the plates, machine movement is reduced since an automatic pipette can move in a linear manner down one row and up another on the 96 well plate.

The location of the well holding each particular oligonucleotides is indicated by row and column. There are eight rows designated A to G and twelve columns designated 1 to 12 in a typical 96 well format plate. Any particular well location is indicated by its "Well No." which is indicated by the combination of the row and the column, e.g. A08 is the well at row A, column 8.

In Table 6-b below, the oligonucleotides of Table 6-a are shown reordered according to the Well No. on their synthesis plate. The order shown in Table 6-b is the actually order as synthesized on an automated synthesizer taking advantage of the preferred placement of the first nucleoside according to the above alignment criteria.

Table 6-b:

Sequences of Oligonucleotides Targeted to CD40 Order by Synthesis Well No.

Well No.		SEQ ID NO:
A01	GACCAGGCGGCAGGACCA	2
A02	AGGTGAGACCAGGCGGCA	3
A03	GCAGAGGCAGACGAACCA	5
A04	GCAAGCAGCCCCAGAGGA	6
A05	GGTCAGCAAGCAGCCCCA	7
A06	GACAGCGGTCAGCAAGCA	8
A07	GATGGACAGCGGTCAGCA	9
A08	GGTGGTTCTGGATGGACA	11

A09	GCAGTGGGTGGTTCTGGA	13
A10	CACAAAGAACAGCACTGA	14
A11	CTGGCACAAAGAACAGCA	15
A12	TCCTGGCTGGCACAAAGA	16
B01	CTGTCCTGGCTGGCACAA	17
B02	ACTCTGTGCAGTCACTCA	21
B03	TTCACCGCAAGGAAGGCA	25
B04	CTCTGTTCCAGGTGTCTA	26
B05	GTGCCAGCCTTCTTCACA	37
B06	TGCAGGACACAGCTCTCA	40
B07	AATCTGCTTGACCCCAAA	43
B08	GTATCAGAAACCCCTGTA	45
B09	GACTGGGCAGGGCTCGCA	49
B10	CATTGGAGAAGAAGCCGA	50
B11	TCGAAAGCAGATGACACA	53
B12	CAGGTCTTTGGTCTCACA	57
C01	TTTTGATAAAGACCAGCA	68
C02	GATCGTCGGGAAAATTGA	72
C03	TGGAGCAGCAGTGTTGGA	75
C04	CGGTTGGCATCCATGTAA	78
C05	CTGTCTCTCCTGCACTGA	80
C06	TCTGGATGGACAGCGGTC	10
C07	CTGGTGGCAGTGTGTCTC	27
C08	GGTGCCCTTCTGCTGGAC	31
C09	ACAGGTGCAGATGGTGTC	35
C10	GAAACCCCTGTAGCAATC	44
C11	TTGGTCTCACAGCTTGTC	56
C12	CTGTTGCACAACCAGGTC	58
D01	GTCTTGTTTGTGCCTGCC	60
D02	CCACAGACAACATCAGTC	61
D03	CTGGGGACCACAGACAAC	62
D04	TCAGCCGATCCTGGGGAC	63
D05	GTCGGGAAAATTGATCTC	71
D06	GGAGCCAGGAAGATCGTC	73
D07	TGGCATCCATGTAAAGTC	77
D08	AACTGCCTGTTTGCCCAC	82
D09	ACTGACTGGGCATAGCTC	84
D10	GTGGGTGGTTCTGGATGG	12
D11	GAAGGCATTCCGTTTCAG	24
D12	GTGTCTGTTTCTGAGGTG	33
E01	TGGTGTCTGTTTCTGAGG	34
E02	GGACACAGCTCTCACAGG	39
E03	GAGCGGTGCAGGACACAG	41
E04	AAGCCGGGCGAGCATGAG	42
E05	GCTCGCAGATGGTATCAG	46
E06	GATGACACATTGGAGAAG	51
E07	GCAGATGACACATTGGAG	52
E08	GTTTGTGCCTGCCTGTTG	59
E09	CACCACCAGGGCTCTCAG	64
E10	ACCAGCACCAAGAGGATG	67
E11	TATTGGTTGGCTTCTTGG	69

E12	GGGTTCCTGCTTGGGGTG	70
F01	GTAAAGTCTCCTGCACTG	76
F02	CTCTTTGCCATCCTCCTG	79
F03	CTTCTGCCTGCACCCCTG	83
F04	CCAGGCGGCAGGACCACT	1
F05	CAGAGGCAGACGAACCAT	4
F06	CTCACCAGTTTCTGTCCT	18
F07	TCACTCACCAGTTTCTGT	19
F08	GTGCAGTCACTCACCAGT	20
F09	CAGTGAACTCTGTGCAGT	22
F10	ATTCCGTTTCAGTGAACT	23
F11	TGGGGTCGCAGTATTTGT	28
F12	GGTTGGGGTCGCAGTATT	29
G01	CTAGGTTGGGGTCGCAGT	30
G02	CTGAGGTGCCCTTCTGCT	32
G03	TTCACAGGTGCAGATGGT	36
G04	TACAGTGCCAGCCTTCTT	38
G05	GCAGGGCTCGCAGATGGT	47
G06	TGGGCAGGGCTCGCAGAT	48
G07	GTCCAAGGGTGACATTTT	54
G08	CACAGCTTGTCCAAGGGT	55
G09	GGGATCACCACCAGGGCT	65
G10	GAGGATGGCAAACAGGAT	66
G11	TGGAGCCAGGAAGATCGT	74
G12	GGTGCAGCCTCACTGTCT	81

EXAMPLE 9: Oligonucleotide Analysis

A. Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates were diluted from the master plate using single and multi-channel robotic pipettors.

B. Alternative Oligonucleotide Analysis

After cleavage from the controlled pore glass support (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides are analyzed by polyacrylamide gel electrophoresis on denaturing gels. Oligonucleotide purity is checked by ^{31}P nuclear

magnetic resonance spectroscopy, and/or by HPLC, as described by Chiang *et al.*, *J. Biol. Chem.* 1991, *266,* 18162.

EXAMPLE 10: Automated Assay of CD40 Oligonucleotide Activity

A. Poly(A)+ mRNA isolation.

Poly(A)+ mRNA was isolated according to Miura *et al.* (*Clin. Chem.*, 1996, *42*, 1758). Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 µl cold PBS. 60 µl lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55 µl of lysate was transferred to Oligo d(T) coated 96 well plates (AGCT Inc., Irvine, CA). Plates were incubated for 60 minutes at room temperature, washed 3 times with 200 ml of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 ml of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C plate for 5 minutes, and the eluate then transferred to a fresh 96-well plate. Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

B. Total RNA isolation

Total mRNA was isolated using an RNEASY 96Ô kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200 mL cold PBS. 100 mL Buffer RLT was added to each well and the plate vigorously agitated for 20 seconds. 100 mL of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96Ô well plate attached to a QIAVACÔ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96Ô plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96Ô plate and the vacuum applied for a period of 15 seconds. The

Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate was then removed from the QIAVACÔ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVACÔ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by pipetting 60 mL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 mL water.

C. RT-PCR Analysis of CD40 mRNA Levels

Quantitation of CD40 mRNA levels was determined by reverse transcriptase polymerase chain reaction (RT-PCR) using the ABI PRISM™_7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time.

As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in RT-PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated.

With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular (six-second) intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from

untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

RT-PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25 ml PCR cocktail (1x TAQMAN™ buffer A, 5.5 mM $MgCl_2$, 300 mM each of dATP, dCTP and dGTP, 600 mM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 U RNAse inhibitor, 1.25 units AMPLITAQ GOLD™, and 12.5 U MuLV reverse transcriptase) to 96 well plates containing 25 ml poly(A) mRNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD™, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

For CD40, the PCR primers were:

forward: 5' CAGAGTTCACTGAAACGGAATGC 3'

(SEQ ID NO:86)

reverse: 5' GGTGGCAGTGTGTCTCTCTGTTC 3' (SEQ ID NO:87), and

PCR probe: 5' *FAM*-TTCCTTGCGGTGAAAGCGAATTCCT-*TAMRA* 3' (SEQ ID NO:88) where *FAM* (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and *TAMRA* (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

For GAPDH, the PCR primers were:

forward: 5' GAAGGTGAAGGTCGGAGTC 3' (SEQ ID NO:89)

reverse: 5' GAAGATGGTGATGGGATTTC 3' (SEQ ID NO:90), and

PCR probe: 5' *JOE*-CAAGCTTCCCGTTCTCAGCC-*TAMRA* 3' (SEQ ID NO. 91)

where *JOE* (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye and *TAMRA* (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXAMPLE 11: Inhibition of CD40 Expression by Phosphorothioate Oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides complementary to mRNA were designed to target different regions of the human CD40 mRNA, using published sequences (GenBank accession number X60592, incorporated herein by reference as SEQ ID NO: 85). The oligonucleotides are shown in Table 7.

Target sites are indicated by the beginning nucleotide numbers, as given in the sequence source reference (X60592), to which the oligonucleotide binds. All compounds in Table 7 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. Data are averages from three experiments.

Table 7:

Inhibition of CD40 mRNA Levels by Phosphorothioate Oligodeoxynucleotides

ISIS#	TARGET SITE	SEQUENCE	% INHIB.	SEQ ID NO.
18623	18	CCAGGCGGCAGGACCA	30.71	1
18624	20	GACCAGGCGGCAGGAC	28.09	2
18625	26	AGGTGAGACCAGGCGG	21.89	3
18626	48	CAGAGGCAGACGAACC	0.00	4
18627	49	GCAGAGGCAGACGAAC	0.00	5
18628	73	GCAAGCAGCCCCAGAG	0.00	6
18629	78	GGTCAGCAAGCAGCCC	29.96	7
18630	84	GACAGCGGTCAGCAAG	0.00	8
18631	88	GATGGACAGCGGTCAG	0.00	9
18632	92	TCTGGATGGACAGCGG	0.00	10
18633	98	GGTGGTTCTGGATGGA	0.00	11
18634	101	GTGGGTGGTTCTGGAT	0.00	12
18635	104	GCAGTGGGTGGTTCTG	0.00	13
18636	152	CACAAAGAACAGCACT	0.00	14
18637	156	CTGGCACAAAGAACAG	0.00	15
18638	162	TCCTGGCTGGCACAAA	0.00	16
18639	165	CTGTCCTGGCTGGCAC	4.99	17
18640	176	CTCACCAGTTTCTGTCC	0.00	18
18641	179	TCACTCACCAGTTTCTG	0.00	19
18642	185	GTGCAGTCACTCACCA	0.00	20
18643	190	ACTCTGTGCAGTCACTC	0.00	21
18644	196	CAGTGAACTCTGTGCA	5.30	22
18645	205	ATTCCGTTTCAGTGAAC	0.00	23
18646	211	GAAGGCATTCCGTTTC	9.00	24
18647	222	TTCACCGCAAGGAAGG	0.00	25
18648	250	CTCTGTTCCAGGTGTCT	0.00	26
18649	267	CTGGTGGCAGTGTGTC	0.00	27
18650	286	TGGGGTCGCAGTATTT	0.00	28
18651	289	GGTTGGGGTCGCAGTA	0.00	29
18652	292	CTAGGTTGGGGTCGCA	0.00	30
18653	318	GGTGCCCTTCTGCTGG	19.67	31
18654	322	CTGAGGTGCCCTTCTGC	15.63	32
18655	332	GTGTCTGTTTCTGAGGT	0.00	33
18656	334	TGGTGTCTGTTTCTGAG	0.00	34
18657	345	ACAGGTGCAGATGGTG	0.00	35
18658	348	TTCACAGGTGCAGATG	0.00	36

	18659	360	GTGCCAGCCTTCTTCAC	5.67	37
	18660	364	TACAGTGCCAGCCTTCT	7.80	38
	18661	391	GGACACAGCTCTCACA	0.00	39
	18662	395	TGCAGGACACAGCTCT	0.00	40
	18663	401	GAGCGGTGCAGGACAC	0.00	41
	18664	416	AAGCCGGGCGAGCATG	0.00	42
	18665	432	AATCTGCTTGACCCCA	5.59	43
	18666	446	GAAACCCCTGTAGCAA	0.10	44
	18667	452	GTATCAGAAACCCCTG	0.00	45
	18668	463	GCTCGCAGATGGTATC	0.00	46
	18669	468	GCAGGGCTCGCAGATG	34.05	47
	18670	471	TGGGCAGGGCTCGCAG	0.00	48
	18671	474	GACTGGGCAGGGCTCG	2.71	49
	18672	490	CATTGGAGAAGAAGCC	0.00	50
	18673	497	GATGACACATTGGAGA	0.00	51
	18674	500	GCAGATGACACATTGG	0.00	52
	18675	506	TCGAAAGCAGATGACA	0.00	53
	18676	524	GTCCAAGGGTGACATT	8.01	54
	18677	532	CACAGCTTGTCCAAGG	0.00	55
	18678	539	TTGGTCTCACAGCTTGT	0.00	56
	18679	546	CAGGTCTTTGGTCTCAC	6.98	57
	18680	558	CTGTTGCACAACCAGG	18.76	58
	18681	570	GTTTGTGCCTGCCTGTT	2.43	59
	18682	575	GTCTTGTTTGTGCCTGC	0.00	60
	18683	590	CCACAGACAACATCAG	0.00	61
	18684	597	CTGGGGACCACAGACA	0.00	62
	18685	607	TCAGCCGATCCTGGGG	0.00	63
	18686	621	CACCACCAGGGCTCTC	23.31	64
	18687	626	GGGATCACCACCAGGG	0.00	65
	18688	657	GAGGATGGCAAACAGG	0.00	66
	18689	668	ACCAGCACCAAGAGGA	0.00	67
	18690	679	TTTTGATAAAGACCAG	0.00	68
	18691	703	TATTGGTTGGCTTCTTG	0.00	69
	18692	729	GGGTTCCTGCTTGGGG	0.00	70
	18693	750	GTCGGGAAAATTGATC	0.00	71
	18694	754	GATCGTCGGGAAAATT	0.00	72
	18695	765	GGAGCCAGGAAGATCG	0.00	73
	18696	766	TGGAGCCAGGAAGATC	0.00	74
	18697	780	TGGAGCAGCAGTGTTG	0.00	75
	18698	796	GTAAAGTCTCCTGCAC	0.00	76
	18699	806	TGGCATCCATGTAAAG	0.00	77
	18700	810	CGGTTGGCATCCATGT	0.00	78
	18701	834	CTCTTTGCCATCCTCCT	4.38	79
	18702	861	CTGTCTCTCCTGCACTG	0.00	80
	18703	873	GGTGCAGCCTCACTGT	0.00	81
	18704	910	AACTGCCTGTTTGCCCA	33.89	82
	18705	954	CTTCTGCCTGCACCCCT	0.00	83
	18706	976	ACTGACTGGGCATAGC	0.00	84

As shown in Table 7, SEQ ID NOS: 1, 2, 7, 47 and 82 demonstrated at least 25% inhibition of CD40 expression and are therefore preferred compounds of the invention.

EXAMPLE 12: Inhibition of CD40 Expression by Phosphorothioate 2'-MOE Gapmer Oligonucleotides

In accordance with the present invention, a second series of oligonucleotides complementary to mRNA were designed to target different regions of the human CD40 mRNA, using published sequence X60592. The oligonucleotides are shown in Table 8. Target sites are indicated by the beginning or initial nucleotide numbers, as given in the sequence source reference (X60592), to which the oligonucleotide binds.

All compounds in Table 8 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings." The wings are composed of 2'-O-(2-methoxyethyl) (2'-MOE) nucleotides. The intersugar (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data are averaged from three experiments.

Table 8:

Inhibition of CD40 mRNA Levels by Chimeric Phosphorothioate Oligonucleotides

ISIS#	TARGET	SEQUENCE	% Inhibition	SEQ ID
19211	18	CCAGGCGGCAGGACCA	75.71	1
19212	20	GACCAGGCGGCAGGA	77.23	2
19213	26	AGGTGAGACCAGGCG	80.82	3
19214	48	CAGAGGCAGACGAAC	23.68	4
19215	49	GCAGAGGCAGACGAA	45.97	5
19216	73	GCAAGCAGCCCCAGAG	65.80	6
19217	78	GGTCAGCAAGCAGCCC	74.73	7
19218	84	GACAGCGGTCAGCAAG	67.21	8
19219	88	GATGGACAGCGGTCAG	65.14	9
19220	92	TCTGGATGGACAGCGG	78.71	10
19221	98	GGTGGTTCTGGATGGA	81.33	11
19222	101	GTGGGTGGTTCTGGAT	57.79	12
19223	104	GCAGTGGGTGGTTCTG	73.70	13
19224	152	CACAAAGAACAGCACT	40.25	14
19225	156	CTGGCACAAAGAACAG	60.11	15

	19226	162	TCCTGGCTGGCACAAA	10.18	16
	19227	165	CTGTCCTGGCTGGCAC	24.37	17
	19228	176	CTCACCAGTTTCTGTC	22.30	18
	19229	179	TCACTCACCAGTTTCT	40.64	19
5	19230	185	GTGCAGTCACTCACCA	82.04	20
	19231	190	ACTCTGTGCAGTCACT	37.59	21
	19232	196	CAGTGAACTCTGTGCA	40.26	22
	19233	205	ATTCCGTTTCAGTGAA	56.03	23
	19234	211	GAAGGCATTCCGTTTC	32.21	24
10	19235	222	TTCACCGCAAGGAAGG	61.03	25
	19236	250	CTCTGTTCCAGGTGTCT	62.19	26
	19237	267	CTGGTGGCAGTGTGTC	70.32	27
	19238	286	TGGGGTCGCAGTATTT	0.00	28
	19239	289	GGTTGGGGTCGCAGTA	19.40	29
15	19240	292	CTAGGTTGGGGTCGCA	36.32	30
	19241	318	GGTGCCCTTCTGCTGG	78.91	31
	19242	322	CTGAGGTGCCCTTCTG	69.84	32
	19243	332	GTGTCTGTTTCTGAGG	63.32	33
	19244	334	TGGTGTCTGTTTCTGA	42.83	34
20	19245	345	ACAGGTGCAGATGGTG	73.31	35
	19246	348	TTCACAGGTGCAGATG	47.72	36
	19247	360	GTGCCAGCCTTCTTCA	61.32	37
	19248	364	TACAGTGCCAGCCTTC	46.82	38
	19249	391	GGACACAGCTCTCACA	0.00	39
25	19250	395	TGCAGGACACAGCTCT	52.05	40
	19251	401	GAGCGGTGCAGGACAC	50.15	41
	19252	416	AAGCCGGGCGAGCATG	32.36	42
	19253	432	AATCTGCTTGACCCCA	0.00	43
	19254	446	GAAACCCCTGTAGCAA	0.00	44
30	19255	452	GTATCAGAAACCCCTG	36.13	45
	19256	463	GCTCGCAGATGGTATC	64.65	46
	19257	468	GCAGGGCTCGCAGATG	74.95	47
	19258	471	TGGGCAGGGCTCGCAG	0.00	48
	19259	474	GACTGGGCAGGGCTCG	82.00	49
35	19260	490	CATTGGAGAAGAAGCC	41.31	50
	19261	497	GATGACACATTGGAGA	13.81	51
	19262	500	GCAGATGACACATTGG	78.48	52
	19263	506	TCGAAAGCAGATGACA	59.28	53
	19264	524	GTCCAAGGGTGACATT	70.99	54
40	19265	532	CACAGCTTGTCCAAGG	0.00	55
	19266	539	TTGGTCTCACAGCTTG	45.92	56
	19267	546	CAGGTCTTTGGTCTCA	63.95	57
	19268	558	CTGTTGCACAACCAGG	82.32	58
	19269	570	GTTTGTGCCTGCCTGTT	70.10	59
45	19270	575	GTCTTGTTTGTGCCTGC	68.95	60
	19271	590	CCACAGACAACATCAG	11.22	61
	19272	597	CTGGGGACCACAGACA	9.04	62
	19273	607	TCAGCCGATCCTGGGG	0.00	63
	19274	621	CACCACCAGGGCTCTC	23.08	64
50	19275	626	GGGATCACCACCAGGG	57.94	65
	19276	657	GAGGATGGCAAACAG	49.14	66

	19277	668	ACCAGCACCAAGAGG	3.48	67
	19278	679	TTTTGATAAAGACCAG	30.58	68
	19279	703	TATTGGTTGGCTTCTTG	49.26	69
	19280	729	GGGTTCCTGCTTGGGG	13.95	70
5	19281	750	GTCGGGAAAATTGATC	54.78	71
	19282	754	GATCGTCGGGAAAATT	0.00	72
	19283	765	GGAGCCAGGAAGATC	69.47	73
	19284	766	TGGAGCCAGGAAGATC	54.48	74
	19285	780	TGGAGCAGCAGTGTTG	15.17	75
10	19286	796	GTAAAGTCTCCTGCAC	30.62	76
	19287	806	TGGCATCCATGTAAAG	65.03	77
	19288	810	CGGTTGGCATCCATGT	34.49	78
	19289	834	CTCTTTGCCATCCTCCT	41.84	79
	19290	861	CTGTCTCTCCTGCACT	25.68	80
15	19291	873	GGTGCAGCCTCACTGT	76.27	81
	19292	910	AACTGCCTGTTTGCCC	63.34	82
	19293	954	CTTCTGCCTGCACCCC	0.00	83
	19294	976	ACTGACTGGGCATAGC	11.55	84

20 As shown in Table 8, SEQ ID NOS: 1, 2, 3, 6, 7, 8, 9, 10, 11, 12, 13, 15, 20, 23, 25, 26, 27, 31, 32, 33, 35, 37, 40, 41, 46, 47, 49, 52, 53, 54, 57, 58, 59, 60, 65, 71, 73, 74, 77, 81 and 82 demonstrated at least 50% inhibition of CD40 expression and are therefore preferred compounds of the invention.

25 **EXAMPLE 13: Oligonucleotide-Sensitive Sites of the CD40 Target Nucleic Acid**

As the data presented in the preceding two Examples shows, several sequences were present in preferred compounds of two distinct oligonucleotide chemistries. Specifically, compounds having SEQ ID NOS: 1, 2, 7, 47 and 82 are preferred in both instances. These compounds map to different regions of the CD40 transcript but

30 nevertheless define accessible sites of the target nucleic acid.

For example, SEQ ID NOS: 1 and 2 overlap each other and both map to the 5-untranslated region (5'-UTR) of CD40. Accordingly, this region of CD40 is particularly preferred for modulation via sequence-based technologies. Similarly, SEQ ID NOS: 7 and 47 map to the open reading frame of CD40, whereas SEQ ID NO: 82 maps to the 3'-

35 untranslated region (3'-UTR). Thus, the ORF and 3'-UTR of CD40 may be targeted by sequence-based technologies as well.

The reverse complements of the active CD40 compounds are easily determined by those skilled in the art and may be assembled to yield nucleotide sequences corresponding

to accessible sites on the target nucleic acid. For example, the assembled reverse complement of SEQ ID NOS: 1 and 2 is represented below as SEQ ID NO:92:

5'- AGTGGTCCTGCCGCCTGGTC -3' SEQ ID NO:92

TCACCAGGACGGCGGACC -5' SEQ ID NO:1

ACCAGGACGGCGGACCAG -5' SEQ ID NO:2

Through multiple iterations of the process of the invention, more extensive "footprints" are generated. A library of this information is compiled and may be used by those skilled in the art in a variety of sequence-based technologies to study the molecular and biological functions of CD40 and to investigate or confirm its role in various diseases and disorders.

EXAMPLE 14: Site Selection Program

In a preferred embodiment of the invention, illustrated in Figure 20, an application is deployed which facilitates the selection process for determining the target positions of the oligos to be synthesized, or "sites." This program is written using a three-tiered object-oriented approach. All aspects of the software described, therefore, are tightly integrated with the relational database. For this reason, explicit database read and write steps are not shown. It should be assumed that each step described includes database access. The description below illustrates one way the program can be used. The actual interface allows users to skip from process to process at will, in any order.

Before running the site picking program, the target must have all relevant properties computed as described previously and indicated in process step **2204**. When the site picking program is launched at process step **2206** the user is presented with a panel showing targets which have previously been selected and had their properties calculated. The user selects one target to work with at process step **2208** and proceeds to decide if any derived properties will be needed at process step **2210**. Derived properties are calculated by performing mathematical operations on combinations of pre-calculated properties as defined by the user at process step **2212**.

The derived properties are made available as peers with all the pre-calculated properties. The user selects one of the properties to view plotted versus target position at process step **2214**. This graph is shown above a linear representation of the target. The

horizontal or position axis of both the graph and target are linked and scalable by the user. The zoom range goes from showing the full target length to showing individual target bases as letters and individual property points. The user next selects a threshold value below or above which all sites will be eliminated from future consideration at process step **2216**. The user decides whether to eliminate more sites based on any other properties at process step **2218**. If they choose to eliminate more, they return to pick another property to display at process step **2214** and threshold at process step **2216**.

After eliminating sites, the user selects from the remaining list by choosing any property at process step **2220** and then choosing a manual or automatic selection technique at process step **2222**. In the automatic technique, the user decides whether they want to pick from maxima or minima and the number of maxima or minima to be selected as sites at process step **2224**. The software automatically finds and picks the points. When picking manually the user must decide if they wish to use automatic peak finding at process step **2226**. If the user selects automatic peak finding, then user must click on the graphed property with the mouse at process step **2236**. The nearest maxima or minima, depending on the modifier key held down, to the selected point will be picked as the site. Without the peak finding option, the user must pick a site at process step **2238** by clicking on its position on the linear representation of target.

Each time a site, or group of sites, is picked, a dynamic property is calculated for all possible sites (not yet eliminated) at process step **2230**. This property indicates the nearness of the site to a picked site allowing the user to pick sites in subsequent iterations based on target coverage. After new sites are picked, the user determines if the desired number of sites has been picked. If too few sites have been picked the user returns to pick more **2220**. If too many sites have been picked, the user may eliminate them by selecting and deleting them on the target display at process step **2234**. If the correct number of sites is picked, and the user is satisfied with the set of picked sites, the user registers these sites to the database along with their name, notebook number, and page number at process step **2238**. The database time stamps this registration event.

EXAMPLE 15: Site Selection Program

In a preferred embodiment of the invention, illustrated in Figure 21, an application is deployed which facilitates the assignment of specific chemical structure to the complement of the sequence of the sites previously picked and facilitates the registration and ordering of these now fully defined antisense compounds. This program is written using a three-tiered object-oriented approach. All aspects of the software described, therefore, are tightly integrated with the relational database. For this reason, explicit database read and write steps are not shown, it being understood that each step described also includes appropriate database read/write access.

To begin using the oligonucleotide chemistry assignment program, the user launches it at process step **2302**. The user then selects from the previously selected sets of oligonucleotides at process step **2304**, registered to the database in site picker's process step **2238**. Next, the user must decide whether to manually assign the chemistry a base at a time, or run the sites through a template at process step **2306**. If the user chooses to use a template, they must determine if a desired template is available at process step **2308**. If a template is not available with the desired chemistry modifications and the correct length, the user can define one at process step **2314**.

To define a template, the user must select the length of the oligonucleotide the template is to define. This oligonucleotide is then represented as a bar with selectable regions. The user sets the number of regions on the oligonucleotide, and the positions and lengths of these regions by dragging them back and forth on the bar. Each region is represented by a different color.

For each region, the user defines the chemistry modifications for the sugars, the linkers, and the heterocycles at each base position in the region. At least four heterocycle chemistries must be given, one for each of the four possible base types (A, G, C or T or U) in the site sequence the template will be applied to. A user interface is provided to select these chemistries which show the molecular structure of each component selected and its modification name. By pushing on a pop-up list next to each of the pictures, the user may choose from a list of structures and names, those possible to put in this place. For example, the heterocycle that represents the base type G is shown as a two dimensional structure diagram. If the user clicks on the pop-up list, a row of other possible structures

and names is shown. The user drags the mouse to the desired chemistry and releases the mouse. Now the newly selected molecule is displayed as the choice for G type heterocycle modifications.

Once the user has created a template, or selected an existing one, the software applies the template at process step **2312** to each of the complements of the sites in the list. When the templates are applied, it is possible that chemistries will be defined which are impossible to make with the chemical precursors presently used on the automatic synthesizer. To check this, a database is maintained of all precursors previously designed, and their availability for automated synthesis. When the templates are applied, the resulting molecules are tested at process step **2316** against this database to see if they are readily synthesized.

If a molecule is not readily synthesized, it is added to a list that the user inspects. At process step **2318**, the user decides whether to modify the chemistry to make it compatible with the currently recognized list of available chemistries or to ignore it. To modify a chemistry, the user must use the base at a time interface at process step **2322**. The user can also choose to go directly to this step, bypassing templates all together at process step **2306**.

The base at a time interface at process step **2322** is very similar to the template editor at process step **2314** except that instead of specifying chemistries for regions, they are defined one base at a time. This interface also differs in that it dynamically checks to see if the design is readily synthesized as the user makes selections. In other words, each choice made limits the choices the software makes available on the pop-up selection lists. To accommodate this function, an additional choice is made available on each pop-up of "not defined." For example, this allows the user to inhibit linker choice from restricting the sugar choices by first setting the linker to "not defined." The user would then pick the sugar, and then pick from the remaining linker choices available.

Once all of the sites on the list are assigned chemistries or dropped, they are registered at process step **2324** to a commercial chemical structure database. Registering to this database makes sure the structure is unique, assigns it a new identifier if it is unique, and allows future structure and substructure searching by creating various hash-tables. The compound definition is also stored at process step **2326** to various hash tables

referred to as chemistry/position tables. These allow antisense compound searching and categorization based on oligonucleotide chemistry modification sequences and equivalent base sequences.

The results of the registration are displayed at process step **2328** with the new IDs if they are new compounds and with the old IDs if they have been previously registered. The user next selects which of the compounds processed they wish to order for synthesis at process step **2330** and registers an order list at process step **2332** by including scientist name, notebook number and page number. The database time-stamps this entry. The user may then choose at process step **2334**, to quit the program at process step **2338**, go back to the beginning and choose a new site list to work with process step **2304**, or start the oligonucleotide ordering interface at process step **2336**.

EXAMPLE 16: Gene Walk to Optimize Oligonucleotide Sequence

A gene walk is executed using a CD40 antisense oligonucleotide having SEQ ID NO:15 (5'-CTGGCACAAAGAACAGCA-3'). In effecting this gene walk, the following parameters are used:

Gene Walk Parameter	**Entered value**
Oligonucleotide Sequence ID:	15
Name of Gene Target:	CD40
Scope of Gene Walk:	20
Sequence Shift Increment:	1

Entering these values and effecting the gene walk centered on SEQ ID NO: 15 automatically generates the following new oligonucleotides:

Table 9:

Oligonucleotide Generated By Gene Walk

SEQ ID	Sequence
93	GAACAGCACTGACTG
94	AGAACAGCACTGACT
95	AAGAACAGCACTGAC
96	AAAGAACAGCACTGA
97	CAAAGAACAGCACTG

98	ACAAAGAACAGCACT
14	CACAAAGAACAGCAC
100	GCACAAAGAACAGCA
101	GGCACAAAGAACAGC
102	TGGCACAAAGAACAG
15	CTGGCACAAAGAACA
103	GCTGGCACAAAGAAC
104	GGCTGGCACAAAGAA
105	TGGCTGGCACAAAGA
106	CTGGCTGGCACAAAG
107	CCTGGCTGGCACAAA
16	TCCTGGCTGGCACAA
109	GTCCTGGCTGGCACA
110	TGTCCTGGCTGGCACA
17	CTGTCCTGGCTGGCAC
112	TCTGTCCTGGCTGGCA

The list shown above contains 20 oligonucleotide sequences directed against the CD40 nucleic acid sequence. They are ordered by the position along the CD40 sequence at which the 5' terminus of each oligonucleotide hybridizes. Thus, the first ten oligonucleotides are single-base frame shift sequences directed against the CD40 sequence upstream of compound SEQ ID NO: 15 and the latter ten are single-base frame shift sequences directed against the CD40 sequence downstream of compound SEQ ID NO: 15.

EXAMPLE 17: Automated Assay of RhoC Oligonucleotide Activity

RhoC, a member of the Rho subfamily of small GTPases, is a protein that has been shown to be involved in a diverse set of signaling pathways including the ultimate regulation of the dynamic organization of the cytoskeleton.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes.

RhoC probes and primers were designed to hybridize to the human RhoC sequence, using published sequence information (GenBank accession number L25081, incorporated herein by reference as SEQ ID NO:113).

For RhoC the PCR primers were:

forward primer: TGATGTCATCCTCATGTGCTTCT (SEQ ID NO: 114)

reverse primer: CCAGGATGATGGGCACGTT (SEQ ID NO: 115) and the PCR probe was: FAM-CGACAGCCCTGACAGCCTGGAAA-TAMRA (SEQ ID NO: 116) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXAMPLE 18: Antisense inhibition of RhoC expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human RhoC RNA, using published sequences (GenBank accession number L25081, incorporated herein by reference as SEQ ID NO: 113). The oligonucleotides are shown in Table 10. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. L25081), to which the oligonucleotide binds. All compounds in Table 10 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on RhoC mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 10

Inhibition of RhoC mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
25304	5' UTR	4	gagctgagatgaagtcaa	29	117
25305	5' UTR	44	gctgaagttcccaggctg	25	118
25306	5' UTR	47	ccggctgaagttcccagg	42	119
25307	Coding	104	ggcaccatccccaacgat	81	120
25308	Coding	105	aggcaccatccccaacga	81	121
25309	Coding	111	tcccacaggcaccatccc	70	122
25310	Coding	117	aggtcttcccacaggcac	40	123
25311	Coding	127	atgaggaggcaggtcttc	41	124
25312	Coding	139	ttgctgaagacgatgagg	23	125
25313	Coding	178	tcaaagacagtagggacg	0	126
25314	Coding	181	ttctcaaagacagtaggg	2	127
25315	Coding	183	agttctcaaagacagtag	38	128
25316	Coding	342	tgttttccaggctgtcag	59	129

	25317	Coding	433	tcgtcttgcctcaggtcc	79	130
	25318	Coding	439	gtgtgctcgtcttgcctc	67	131
	25319	Coding	445	ctcctggtgtgctcgtct	67	132
	25320	Coding	483	cagaccgaacgggctcct	65	133
	25321	Coding	488	ttcctcagaccgaacggg	57	134
	25322	Coding	534	actcaaggtagccaaagg	33	135
	25323	Coding	566	ctcccgcactccctcctt	91	136
	25324	Coding	575	ctcaaacacctcccgcac	34	137
	25325	Coding	581	ggccatctcaaacacctc	64	138
	25326	Coding	614	cttgttcttgcggacctg	72	139
	25327	Coding	625	cccctccgacgcttgttc	66	140
	25328	3' UTR	737	gtatggagccctcaggag	60	141
	25329	3' UTR	746	gagccttcagtatggagc	63	142
	25330	3' UTR	753	gaaaatggagccttcagt	24	143
	25331	3' UTR	759	ggaactgaaaatggagcc	2	144
	25332	3' UTR	763	ggagggaactgaaaatgg	13	145
	25333	3' UTR	766	gcaggagggaactgaaaa	27	146
	25334	3' UTR	851	agggcagggcataggcgt	31	147
	25335	3' UTR	854	ggaagggcagggcatagg	21	148
	25336	3' UTR	859	catgaggaagggcagggc	0	149
	25337	3' UTR	920	taaagtgctggtgtgtga	39	150
	25338	3' UTR	939	cctgtgagccagaagtgt	69	151
	25339	3' UTR	941	ttcctgtgagccagaagt	69	152
	25340	3' UTR	945	cactttcctgtgagccag	82	153
	25341	3' UTR	948	agacactttcctgtgagc	69	154
	25342	3' UTR	966	actctgggtccctactgc	20	155
	25343	3' UTR	992	tgcagaaacaactccagg	0	156

Example 19: Antisense inhibition of RhoC expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human RhoC were synthesized. The oligonucleotide sequences are shown in Table 11. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession No. L25081), to which the oligonucleotide binds.

All compounds in Table 11 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE) nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the

oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 11

Inhibition of RhoC mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
25344	5' UTR	4	gagctgagatgaagtcaa	0	117
25345	5' UTR	44	gctgaagttcccaggctg	35	118
25346	5' UTR	47	ccggctgaagttcccagg	53	119
25347	Coding	104	ggcaccatccccaacgat	50	120
25348	Coding	105	aggcaccatccccaacga	56	121
25349	Coding	111	tcccacaggcaccatccc	4	122
25350	Coding	117	aggtcttcccacaggcac	11	123
25351	Coding	127	atgaggaggcaggtcttc	6	124
25352	Coding	139	ttgctgaagacgatgagg	15	125
25353	Coding	178	tcaaagacagtagggacg	32	126
25354	Coding	181	ttctcaaagacagtaggg	7	127
25355	Coding	183	agttctcaaagacagtag	39	128
25356	Coding	342	tgttttccaggctgtcag	59	129
25357	Coding	433	tcgtcttgcctcaggtcc	48	130
25358	Coding	439	gtgtgctcgtcttgcctc	36	131
25359	Coding	445	ctcctggtgtgctcgtct	61	132
25360	Coding	483	cagaccgaacgggctcct	50	133
25361	Coding	488	ttcctcagaccgaacggg	14	134
25362	Coding	534	actcaaggtagccaaagg	32	135
25363	Coding	566	ctcccgcactccctcctt	21	136
25364	Coding	575	ctcaaacacctcccgcac	9	137
25365	Coding	581	ggccatctcaaacacctc	66	138
25366	Coding	614	cttgttcttgcggacctg	61	139
25367	Coding	625	cccctccgacgcttgttc	0	140
25368	3' UTR	737	gtatggagccctcaggag	28	141
25369	3' UTR	746	gagccttcagtatggagc	32	142
25370	3' UTR	753	gaaaatggagccttcagt	0	143
25371	3' UTR	759	ggaactgaaaatggagcc	40	144
25372	3' UTR	763	ggagggaactgaaaatgg	45	145
25373	3' UTR	766	gcaggagggaactgaaaa	35	146
25374	3' UTR	851	agggcagggcataggcgt	5	147
25375	3' UTR	854	ggaagggcagggcatagg	0	148

25376	3' UTR	859	catgaggaagggcagggc	0	149
25377	3' UTR	920	taaagtgctggtgtgtga	20	150
25378	3' UTR	939	cctgtgagccagaagtgt	67	151
25379	3' UTR	941	ttcctgtgagccagaagt	61	152
25380	3' UTR	945	cactttcctgtgagccag	80	153
25381	3' UTR	948	agacactttcctgtgagc	0	154
25382	3' UTR	966	actctgggtccctactgc	0	155
25383	3' UTR	992	tgcagaaacaactccagg	0	156

EXAMPLE 20: Automated Assay of Cellular Inhibitor of Apoptosis-2 Expression Oligonucleotide Activity

Cellular Inhibitor of Apoptosis-2 (also known as c-IAP-2, apoptosis inhibitor 2, API-2, hIAP-1, and MIHC) is a member of the inhibitor of apoptosis (IAP) family of anti-apoptotic proteins which interfere with the transmission of intracellular death signals.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. Cellular Inhibitor of Apoptosis-2 probes and primers were designed to hybridize to the human Cellular Inhibitor of Apoptosis-2 sequence, using published sequence information (GenBank accession number U37546, incorporated herein by reference as SEQ ID NO:157).

For Cellular Inhibitor of Apoptosis-2 the PCR primers were:
forward primer: GGACTCAGGTGTTGGGAATCTG (SEQ ID NO: 158)
reverse primer: CAAGTACTCACACCTTGGAAACCA (SEQ ID NO: 159) and the PCR probe was: FAM-AGATGATCCATGGGTTCAACATGCCAA-TAMRA (SEQ ID NO: 160) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXAMPLE 21: Antisense inhibition of Cellular Inhibitor of Apoptosis-2 expression-phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human Cellular Inhibitor of Apoptosis-2 RNA, using published sequences (GenBank accession number U37546, incorporated herein by reference as SEQ ID NO: 157). The oligonucleotides are shown in Table 12. Target sites

are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. U37546), to which the oligonucleotide binds. All compounds in Table 12 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on Cellular Inhibitor of Apoptosis-2 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 12

Inhibition of Cellular Inhibitor of Apoptosis-2 mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
23412	5' UTR	3	actgaagacattttgaat	62	161
23413	5' UTR	37	cttagaggtacgtaaaat	29	162
23414	5' UTR	49	gcacttttatttcttaga	70	163
23415	5' UTR	62	attttaattagaagcact	0	164
23416	5' UTR	139	accatatttcactgattc	70	165
23417	5' UTR	167	ctaactcaaaggaggaaa	0	166
23418	5' UTR	175	cacaagacctaactcaaa	27	167
23419	5' UTR	268	gctctgctgtcaagtgtt	57	168
23420	5' UTR	303	tgtgtgactcatgaagct	23	169
23421	5' UTR	335	ttcagtggcattcaatca	23	170
23422	5' UTR	357	cttctccaggctactaga	50	171
23423	5' UTR	363	ggtcaacttctccaggct	65	172
23424	5' UTR	437	taaaacccttcacagaag	0	173
23425	5' UTR	525	ttaaggaagaaatacaca	0	174
23426	5' UTR	651	gcatggctttgcttttat	0	175
23427	Coding	768	caaacgtgttggcgcttt	35	176
23428	Coding	830	agcaggaaaagtggaata	0	177
23429	Coding	1015	ttaacggaatttagactc	0	178
23430	Coding	1064	atttgttactgaagaagg	0	179
23431	Coding	1118	agagccacggaaatatcc	9	180
23432	Coding	1168	aaatcttgatttgctctg	7	181
23433	Coding	1231	gtaagtaatctggcattt	0	182
23434	Coding	1323	agcaagccactctgtctc	50	183
23435	Coding	1436	tgaagtgtcttgaagctg	0	184
23436	Coding	1580	tttgacatcatcactgtt	0	185
23437	Coding	1716	tggcttgaacttgacgga	0	186
23438	Coding	1771	tcatctcctgggctgtct	40	187

23439	Coding	1861	gcagcattaatcacagga	0	188
23440	Coding	2007	tttctctctcctcttccc	10	189
23441	Coding	2150	aacatcatgttcttgttc	9	190
23442	Coding	2273	atataacacagcttcagc	0	191
23443	Coding	2350	aattgttcttccactggt	0	192
23444	Coding	2460	aagaaggagcacaatctt	70	193
23445	3' UTR	2604	gaaaccaaattaggataa	12	194
23446	3' UTR	2753	tgtagtgctacctctttt	69	195
23447	3' UTR	2779	ctgaaattttgattgaat	14	196
23448	3' UTR	2795	tacaatttcaataatgct	38	197
23449	3' UTR	2920	gggtctcagtatgctgcc	21	198
23450	3' UTR	3005	ccttcgatgtataggaca	0	199
23451	3' UTR	3040	catgtccctaaaatgtca	0	200

EXAMPLE 22: Antisense inhibition of Cellular Inhibitor of Apoptosis-2 expression-phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human Cellular Inhibitor of Apoptosis-2 were synthesized. The oligonucleotide sequences are shown in Table 13. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. U37546), to which the oligonucleotide binds.

All compounds in Table 13 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 13

Inhibition of Cellular Inhibitor of Apoptosis-2 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
23452	5' UTR	3	actgaagacattttgaat	35	161
23453	5' UTR	37	cttagaggtacgtaaaat	26	162
23454	5' UTR	49	gcacttttatttcttaga	76	163
23455	5' UTR	62	attttaattagaagcact	0	164
23456	5' UTR	139	accatatttcactgattc	0	165
23457	5' UTR	167	ctaactcaaaggaggaaa	5	166
23458	5' UTR	175	cacaagacctaactcaaa	0	167
23459	5' UTR	268	gctctgctgtcaagtgtt	57	168
23460	5' UTR	303	tgtgtgactcatgaagct	67	169
23461	5' UTR	335	ttcagtggcattcaatca	59	170
23462	5' UTR	357	cttctccaggctactaga	0	171
23463	5' UTR	363	ggtcaacttctccaggct	75	172
23464	5' UTR	437	taaaacccttcacagaag	11	173
23465	5' UTR	525	ttaaggaagaaatacaca	0	174
23466	5' UTR	651	gcatggctttgcttttat	46	175
23467	Coding	768	caaacgtgttggcgcttt	47	176
23468	Coding	830	agcaggaaaagtggaata	39	177
23469	Coding	1015	ttaacggaatttagactc	12	178
23470	Coding	1064	atttgttactgaagaagg	34	179
23471	Coding	1118	agagccacggaaatatcc	54	180
23472	Coding	1168	aaatcttgatttgctctg	34	181
23473	Coding	1231	gtaagtaatctggcattt	0	182
23474	Coding	1323	agcaagccactctgtctc	42	183
23475	Coding	1436	tgaagtgtcttgaagctg	0	184
23476	Coding	1580	tttgacatcatcactgtt	57	185
23477	Coding	1716	tggcttgaacttgacgga	23	186
23478	Coding	1771	tcatctcctgggctgtct	66	187
23479	Coding	1861	gcagcattaatcacagga	65	188
23480	Coding	2007	tttctctctcctcttccc	0	189
23481	Coding	2150	aacatcatgttcttgttc	13	190
23482	Coding	2273	atataacacagcttcagc	0	191
23483	Coding	2350	aattgttcttccactggt	60	192
23484	Coding	2460	aagaaggagcacaatctt	65	193
23485	3' UTR	2604	gaaaccaaattaggataa	0	194
23486	3' UTR	2753	tgtagtgctacctctttt	73	195
23487	3' UTR	2779	ctgaaattttgattgaat	4	196

23488	3' UTR	2795	tacaatttcaataatgct	0	197
23489	3' UTR	2920	gggtctcagtatgctgcc	42	198
23490	3' UTR	3005	ccttcgatgtataggaca	71	199
23491	3' UTR	3040	catgtccctaaaatgtca	45	200

EXAMPLE 23: Automated Assay of ELK-1 Oligonucleotide Activity

ELK-1 (also known as p62TCF) is a member of the ternary complex factor (TCF) subfamily of Ets domain proteins and utilizes a bipartite recognition mechanism mediated by both protein-DNA and protein-protein interactions. This results in gene regulation not only by direct DNA binding but also by indirect DNA binding through recruitment by other factors (Rao et al., *Science,* **1989**, *244*, 66-70). The formation of ternary complexes with an array of proteins allows the differential regulation of many genes. The mechanism by which ELK-1 controls various signal transduction pathways involves regulating the activity of the Egr-1, pip92, nur77 and c-fos promoters by binding to the serum response element (SRE) in these promoters in response to extracellular stimuli such as growth factors, mitogens and oncogene products (Sharrocks et al., *Int. J. Biochem. Cell Biol.,* **1997**, *29*, 1371-1387). ELK-1 has also been shown to mediate other functions within the cell including apoptosis.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes.

ELK-1 probes and primers were designed to hybridize to the human ELK-1 sequence, using published sequence information (GenBank accession number M25269, incorporated herein by reference as SEQ ID NO:201).

For ELK-1 the PCR primers were:

forward primer: GCAAGGCAATGGCCACAT (SEQ ID NO: 202)

reverse primer: CTCCTCTGCATCCACCAGCTT (SEQ ID NO: 203) and the PCR probe was: FAM-TCTCCTGGACTTCACGGGATGGTGGT-TAMRA (SEQ ID NO: 204) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXAMPLE 24: Antisense inhibition of ELK-1 expression-phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human ELK-1 RNA, using published sequences (GenBank accession number M25269, incorporated herein by reference as SEQ ID NO: 201). The oligonucleotides are shown in Table 14. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M25269), to which the oligonucleotide binds. All compounds in Table 14 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on ELK-1 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 14

Inhibition of ELK-1 mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
24752	5' UTR	11	cccctgcgtttccctaca	15	205
24753	5' UTR	50	ggtggtggtggcggtggc	29	206
24754	5' UTR	139	ggcgttggcaatgttggc	82	207
24755	5' UTR	167	aagttgaggctgtgtgta	0	208
24756	5' UTR	189	aggccacggacgggtctc	92	209
24757	5' UTR	229	gattgattcgctacgatg	71	210
24758	5' UTR	255	gggatgcggaggagtgcg	74	211
24759	5' UTR	289	agtgctcacgccatccca	22	212
24760	Coding	328	aaactgccacagcgtcac	64	213
24761	Coding	381	gaagtccaggagatgatg	62	214
24762	Coding	395	caccaccatcccgtgaag	88	215
24763	Coding	455	tcttgttcttgcgtagtc	62	216
24764	Coding	512	tgttcttgtcatagtagt	52	217
24765	Coding	527	tcaccttgcggatgatgt	57	218
24766	Coding	582	gagcaccctgcgacctca	72	219
24767	Coding	600	ggcgggcagtcctcagtg	82	220
24768	Coding	787	ggtgaaggtggaatagag	58	221
24769	Coding	993	tccgatttcaggtttggg	55	222
24770	Coding	1110	ttggtggtttctggcaca	67	223

24771	Coding	1132	tggagggacttctggctc	69	224
24772	Coding	1376	gcgtaggaagcagggatg	34	225
24773	Coding	1440	gtgctccagaagtgaatg	64	226
24774	Coding	1498	actggatggaaactggaa	34	227
24775	Coding	1541	ggccatccacgctgatag	74	228
24776	3' UTR	1701	ccaccacaatcagagcat	74	229
24777	3' UTR	1711	gatccccaccccaccaca	16	230
24778	3' UTR	1765	tgttttctgtggaggaga	48	231
24779	3' UTR	1790	aaacagagaagttgtgga	11	232
24780	3' UTR	1802	gggactgacagaaaacag	0	233
24781	3' UTR	1860	ataaataaataaccgcc	18	234
24782	3' UTR	1894	gttaggtcaggctcatcc	56	235
24783	3' UTR	1974	gttctcaagccagacctc	52	236
24784	3' UTR	1992	aataaagaaagaaaggtc	41	237
24785	3' UTR	2006	agggcaggctgagaaata	29	238
24786	3' UTR	2053	cttctactcacatccaaa	54	239
24787	3' UTR	2068	caaaacaaactaactctt	24	240
24788	3' UTR	2080	ggaataataaaacaaaac	40	241
24789	3' UTR	2107	ttcttcctggacccctga	93	242
24790	3' UTR	2161	ccaagggtgtgattcttc	81	243
24791	3' UTR	2200	tgtctgagagaaaggttg	55	244

EXAMPLE 25: Antisense inhibition of ELK-1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human ELK-1 were synthesized. The oligonucleotide sequences are shown in Table 15. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M25269), to which the oligonucleotide binds.

All compounds in Table 15 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 15

Inhibition of ELK-1 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
24792	5' UTR	11	cccctgcgtttccctaca	23	205
24793	5' UTR	50	ggtggtggtggcggtggc	80	206
24794	5' UTR	139	ggcgttggcaatgttggc	91	207
24795	5' UTR	167	aagttgaggctgtgtgta	27	208
24796	5' UTR	189	aggccacggacgggtctc	79	209
24797	5' UTR	229	gattgattcgctacgatg	69	210
24798	5' UTR	255	gggatgcggaggagtgcg	42	211
24799	5' UTR	289	agtgctcacgccatccca	45	212
24800	Coding	328	aaactgccacagcgtcac	57	213
24801	Coding	381	gaagtccaggagatgatg	55	214
24802	Coding	395	caccaccatcccgtgaag	41	215
24803	Coding	455	tcttgttcttgcgtagtc	80	216
24804	Coding	512	tgttcttgtcatagtagt	65	217
24805	Coding	527	tcaccttgcggatgatgt	70	218
24806	Coding	582	gagcaccctgcgacctca	64	219
24807	Coding	600	ggcgggcagtcctcagtg	67	220
24808	Coding	787	ggtgaaggtggaatagag	45	221
24809	Coding	993	tccgatttcaggtttggg	75	222
24810	Coding	1110	ttggtggtttctggcaca	82	223
24811	Coding	1132	tggagggacttctggctc	60	224
24812	Coding	1376	gcgtaggaagcagggatg	49	225
24813	Coding	1440	gtgctccagaagtgaatg	71	226
24814	Coding	1498	actggatggaaactggaa	62	227
24815	Coding	1541	ggccatccacgctgatag	78	228
24816	3' UTR	1701	ccaccacaatcagagcat	54	229
24817	3' UTR	1711	gatccccaccccaccaca	44	230
24818	3' UTR	1765	tgttttctgtggaggaga	74	231
24819	3' UTR	1790	aaacagagaagttgtgga	64	232
24820	3' UTR	1802	gggactgacagaaaacag	16	233
24821	3' UTR	1860	ataaataaataaaccgcc	38	234
24822	3' UTR	1894	gttaggtcaggctcatcc	59	235
24823	3' UTR	1974	gttctcaagccagacctc	62	236
24824	3' UTR	1992	aataaagaaagaaaggtc	35	237
24825	3' UTR	2006	agggcaggctgagaaata	0	238
24826	3' UTR	2053	cttctactcacatccaaa	46	239
24827	3' UTR	2068	caaaacaaactaactctt	38	240
24828	3' UTR	2080	ggaataataaaacaaaac	37	241

24829	3' UTR	2107	ttcttcctggacccctga	71	242
24830	3' UTR	2161	ccaagggtgtgattcttc	88	243
24831	3' UTR	2200	tgtctgagagaaaggttg	65	244

EXAMPLE 26: Automated Assay of Gi alpha proteins Oligonucleotide Activity

G-alpha-11 is a member of the Gq subfamily of G proteins whose primary function is to activate PLC-b isoforms producing second messengers and affecting intracellular calcium stores.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. G-alpha-11 probes and primers were designed to hybridize to the human G-alpha-11 sequence, using published sequence information (GenBank accession number AF011497, incorporated herein by reference as SEQ ID NO:245). For G-alpha-11 the PCR primers were:
forward primer: TGACCACCTTCGAGCATCAG (SEQ ID NO: 246)
reverse primer: CGGTCGTAGCATTCCTGGAT (SEQ ID NO: 247) and the PCR probe was: FAM-TCAGTGCCATCAAGACCCTGTGGGAG-TAMRA (SEQ ID NO: 248) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXAMPLE 27: Antisense inhibition of G-alpha-11 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human G-alpha-11 RNA, using published sequences (GenBank accession number AF011497, incorporated herein by reference as SEQ ID NO: 245). The oligonucleotides are shown in Table 16. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. AF011497), to which the oligonucleotide binds. All compounds in Table 16 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on G-alpha-11 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from

three experiments. If present, "N.D." indicates "no data".

Table 16

Inhibition of G-alpha-11 mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
20576	Coding	1	gatggactccagagtcat	0	249
20577	Coding	6	gccatgatggactccaga	75	250
20578	Coding	9	cacgccatgatggactcc	0	251
20579	Coding	25	ctcatcgctcaggcaaca	61	252
20580	Coding	31	cttcacctcatcgctcag	20	253
20581	Coding	36	gactccttcacctcatcg	15	254
20582	Coding	45	atccgcttggactccttc	17	255
20583	Coding	50	cgttgatccgcttggact	0	256
20584	Coding	61	ctcgatctcggcgttgat	0	257
20585	Coding	77	cccgccgcagctgcttct	58	258
20586	Coding	106	cttgagctcgcgccgggc	31	259
20587	Coding	116	gcagcagcagcttgagct	0	260
20588	Coding	127	gcccgtgccgagcagcag	0	261
20589	Coding	146	acgtgctcttcccgctct	28	262
20590	Coding	159	atctgcttgatgaacgtg	0	263
20591	Coding	162	cgcatctgcttgatgaac	0	264
20592	Coding	184	gtagccggcgccgtggat	1	265
20593	Coding	197	tgtcctcctccgagtagc	0	266
20594	Coding	199	cttgtcctcctccgagta	79	267
20595	Coding	207	aagccgcgcttgtcctcc	56	268
20596	Coding	222	tagacgagcttggtgaag	0	269
20597	Coding	230	tgttctggtagacgagct	0	270
20598	Coding	242	tggcggtgaagatgttct	0	271
20599	Coding	258	cggatcatggcctgcatg	1	272
20600	Coding	271	cgtctccatggcccggat	49	273
20601	Coding	285	tagaggatcttgagcgtc	0	274
20602	Coding	287	tgtagaggatcttgagcg	0	275
20603	Coding	297	tgctcgtacttgtagagg	7	276
20604	Coding	306	gccttgttctgctcgtac	25	277
20605	Coding	309	ttggccttgttctgctcg	0	278
20606	Coding	319	caggagcgcattggcctt	0	279
20607	Coding	340	ctccacgtccacctcccg	69	280
20608	Coding	349	ggtcaccttctccacgtc	27	281
20609	Coding	362	gatgctcgaaggtggtca	33	282
20610	Coding	373	actgacgtactgatgctc	36	283
20611	Coding	382	cttgatggcactgacgta	78	284
20612	Coding	388	cagggtcttgatggcact	0	285

	20613	Coding	409	ctggatgcccgggtcctc	0	286
	20614	Coding	411	tcctggatgcccgggtcc	30	287
	20615	Coding	429	cgcctgcggtcgtagcat	0	288
	20616	Coding	440	gctggtactcgcgcctgc	41	289
	20617	Coding	459	tacttggcagagtcggag	34	290
	20618	Coding	468	gtcaggtagtacttggca	76	291
	20619	Coding	479	ggtcaacgtcggtcaggt	18	292
	20620	Coding	489	gtggcgatgcggtcaacg	1	293
	20621	Coding	503	gcaggtagcccaaggtgg	20	294
	20622	Coding	518	cgtcctgctgggtgggca	40	295
	20623	Coding	544	ggtggtgggcacgcggac	0	296
	20624	Coding	555	tcgatgatgccggtggtg	0	297
	20625	Coding	572	ccaggtcgaaagggtact	0	298
	20626	Coding	578	tgttctccaggtcgaaag	33	299
	20627	Coding	584	agatgatgttctccaggt	0	300
	20628	Coding	591	atccggaagatgatgttc	0	301
	20629	Coding	624	ctccgctccgaccgctgg	56	302
	20630	Coding	634	gatccacttcctccgctc	59	303
	20631	Coding	655	tgtcacgttctcaaagca	0	304
	20632	Coding	663	atgatggatgtcacgttc	0	305
	20633	Coding	671	cgagaaacatgatggatg	0	306
	20634	Coding	682	gctgagggcgacgagaaa	75	307
	20635	Coding	709	cgactccaccaggacttg	40	308
	20636	Coding	726	atccggttctcgttgtcc	22	309
	20637	Coding	728	ccatccggttctcgttgt	19	310
	20638	Coding	744	agggctttgctctcctcc	77	311
	20639	Coding	754	ggtccggaacagggcttt	26	312
	20640	Coding	766	gtaggtgatgatggtccg	0	313
	20641	Coding	787	ggaggagttctggaacca	64	314
	20642	Coding	803	tgaggaagaggatgacgg	0	315
	20643	Coding	818	gcaggtccttcttgttga	6	316
	20644	Coding	831	atcttgtcctccagcagg	4	317
	20645	Coding	842	gcgagtacaggatcttgt	17	318
	20646	Coding	858	aagtagtccaccaggtgc	0	319
	20647	Coding	910	gatgaactcccgcgccgc	52	320
	20648	Coding	935	ggttcaggtccacgaaca	71	321
	20649	Coding	958	gtagatgatcttgtcgct	0	322
	20650	Coding	972	cacgtgaagtgtgagtag	0	323
	20651	Coding	993	atgttctccgtgtcggtg	0	324
	20652	Coding	1014	acggccgcgaacacgaag	6	325
	20653	Coding	1027	gatggtgtccttcacggc	0	326
	20654	Coding	1043	tcaggttcagctgcagga	3	327
	20655	Coding	1059	accagattgtactccttc	0	328

EXAMPLE 28: Antisense inhibition of G-alpha-11 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human G-alpha-11 were synthesized. The oligonucleotide sequences are shown in Table 17. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. AF011497), to which the oligonucleotide binds.

All compounds in Table 17 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 17

Inhibition of G-alpha-11 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
20981	Coding	1	gatggactccagagtcat	0	249
20982	Coding	6	gccatgatggactccaga	0	250
20983	Coding	9	cacgccatgatggactcc	0	251
20984	Coding	25	ctcatcgctcaggcaaca	0	252
20985	Coding	31	cttcacctcatcgctcag	2	253
20986	Coding	36	gactccttcacctcatcg	0	254
20987	Coding	45	atccgcttggactccttc	19	255
20988	Coding	50	cgttgatccgcttggact	15	256
20989	Coding	61	ctcgatctcggcgttgat	0	257
20990	Coding	77	cccgccgcagctgcttct	41	258
20991	Coding	106	cttgagctcgcgccgggc	19	259
20992	Coding	116	gcagcagcagcttgagct	23	260
20993	Coding	127	gcccgtgccgagcagcag	38	261
20994	Coding	146	acgtgctcttcccgctct	34	262
20995	Coding	159	atctgcttgatgaacgtg	56	263

	20996	Coding	162	cgcatctgcttgatgaac	31	264
	20997	Coding	184	gtagccggcgccgtggat	0	265
	20998	Coding	197	tgtcctcctccgagtagc	42	266
	20999	Coding	199	cttgtcctcctccgagta	0	267
	21000	Coding	207	aagccgcgcttgtcctcc	73	268
	21001	Coding	222	tagacgagcttggtgaag	0	269
	21002	Coding	230	tgttctggtagacgagct	61	270
	21003	Coding	242	tggcggtgaagatgttct	14	271
	21004	Coding	258	cggatcatggcctgcatg	84	272
	21005	Coding	271	cgtctccatggcccggat	70	273
	21006	Coding	285	tagaggatcttgagcgtc	39	274
	21007	Coding	287	tgtagaggatcttgagcg	28	275
	21008	Coding	297	tgctcgtacttgtagagg	70	276
	21009	Coding	306	gccttgttctgctcgtac	76	277
	21010	Coding	309	ttggccttgttctgctcg	0	278
	21011	Coding	319	caggagcgcattggcctt	87	279
	21012	Coding	340	ctccacgtccacctcccg	0	280
	21013	Coding	349	ggtcaccttctccacgtc	69	281
	21014	Coding	362	gatgctcgaaggtggtca	0	282
	21015	Coding	373	actgacgtactgatgctc	69	283
	21016	Coding	382	cttgatggcactgacgta	32	284
	21017	Coding	388	cagggtcttgatggcact	19	285
	21018	Coding	409	ctggatgcccgggtcctc	63	286
	21019	Coding	411	tcctggatgcccgggtcc	56	287
	21020	Coding	429	cgcctgcggtcgtagcat	73	288
	21021	Coding	440	gctggtactcgcgcctgc	68	289
	21022	Coding	459	tacttggcagagtcggag	50	290
	21023	Coding	468	gtcaggtagtacttggca	13	291
	21024	Coding	479	ggtcaacgtcggtcaggt	64	292
	21025	Coding	489	gtggcgatgcggtcaacg	52	293
	21026	Coding	503	gcaggtagcccaaggtgg	52	294
	21027	Coding	518	cgtcctgctgggtgggca	0	295
	21028	Coding	544	ggtggtgggcacgcggac	81	296
	21029	Coding	555	tcgatgatgccggtggtg	48	297
	21030	Coding	572	ccaggtcgaaagggtact	61	298
	21031	Coding	578	tgttctccaggtcgaaag	0	299
	21032	Coding	584	agatgatgttctccaggt	0	300
	21033	Coding	591	atccggaagatgatgttc	0	301
	21034	Coding	624	ctccgctccgaccgctgg	59	302
	21035	Coding	634	gatccacttcctccgctc	17	303
	21036	Coding	655	tgtcacgttctcaaagca	9	304
	21037	Coding	663	atgatggatgtcacgttc	41	305
	21038	Coding	671	cgagaaacatgatggatg	0	306

	21039	Coding	682	gctgagggcgacgagaaa	11	307
	21040	Coding	709	cgactccaccaggacttg	0	308
	21041	Coding	726	atccggttctcgttgtcc	67	309
	21042	Coding	728	ccatccggttctcgttgt	30	310
	21043	Coding	744	agggctttgctctcctcc	61	311
	21044	Coding	754	ggtccggaacagggcttt	72	312
	21045	Coding	766	gtaggtgatgatggtccg	68	313
	21046	Coding	787	ggaggagttctggaacca	54	314
	21047	Coding	803	tgaggaagaggatgacgg	23	315
	21048	Coding	818	gcaggtccttcttgttga	0	316
	21049	Coding	831	atcttgtcctccagcagg	39	317
	21050	Coding	842	gcgagtacaggatcttgt	74	318
	21051	Coding	858	aagtagtccaccaggtgc	36	319
	21052	Coding	910	gatgaactcccgcgccgc	67	320
	21053	Coding	935	ggttcaggtccacgaaca	37	321
	21054	Coding	958	gtagatgatcttgtcgct	64	322
	21055	Coding	972	cacgtgaagtgtgagtag	37	323
	21056	Coding	993	atgttctccgtgtcggtg	0	324
	21057	Coding	1014	acggccgcgaacacgaag	0	325
	21058	Coding	1027	gatggtgtccttcacggc	69	326
	21059	Coding	1043	tcaggttcagctgcagga	0	327
	21060	Coding	1059	accagattgtactccttc	0	328

EXAMPLE 29: Automated Assay of AKT-1 Oligonucleotide Activity

Akt-1 (also known as PKB alpha and RAC-PK alpha) is a member of the AKT/PKB family of serine/threonine kinases and has been shown to be involved in a diverse set of signaling pathways.

Oligonucleotides were designed as described in Example 2, synthesized as described in Examples 3 through 8, analyzed as described in Examples 9 and assayed as described in Example 10 except for target specific primer and probes. AKT-1 probes and primers were designed to hybridize to the human AKT-1 sequence, using published sequence information (GenBank accession number M63167, incorporated herein by reference as SEQ ID NO:329). For Akt-1 the PCR primers were:
forward primer: CGTGACCATGAACGAGTTTGA (SEQ ID NO: 330)
reverse primer: CAGGATCACCTTGCCGAAA (SEQ ID NO: 331) and the PCR probe was: FAM-CTGAAGCTGCTGGGCAAGGGCA-TAMRA (SEQ ID NO: 332) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

EXMAMPLE 30: Antisense inhibition of Akt-1 expression- phosphorothioate oligodeoxynucleotides

In accordance with the present invention, a series of oligonucleotides were designed to target different regions of the human Akt-1 RNA, using published sequences (GenBank accession number M63167, incorporated herein by reference as SEQ ID NO: 329). The oligonucleotides are shown in Table 18. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M63167), to which the oligonucleotide binds. All compounds in Table 18 are oligodeoxynucleotides with phosphorothioate backbones (internucleoside linkages) throughout. The compounds were analyzed for effect on Akt-1 mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from three experiments. If present, "N.D." indicates "no data".

Table 18

Inhibition of Akt-1 mRNA levels by phosphorothioate oligodeoxynucleotides

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
28880	5' UTR	4	ccctgtgccctgtcccag	55	333
28881	5' UTR	27	cctaagcccctggtgaca	15	334
28882	5' UTR	62	ctttgacttctttgaccc	68	335
28883	5' UTR	70	ggcagccccttgacttc	53	336
28884	Coding	213	caaccctccttcacaata	24	337
28885	Coding	234	tactcccctcgtttgtgc	0	338
28886	Coding	281	tgccatcattcttgagga	65	339
28887	Coding	293	agccaatgaaggtgccat	67	340
28888	Coding	352	cacagagaagttgttgag	22	341
28889	Coding	496	agtctggatggcggttgt	49	342
28890	Coding	531	tcctcctcctcctgcttc	9	343
28891	Coding	570	cctgagttgtcactgggt	49	344
28892	Coding	666	ccgaaagtgcccttgccc	56	345
28893	Coding	744	gccacgatgacttccttc	60	346
28894	Coding	927	cggtcctcggagaacaca	0	347
28895	Coding	990	acgttcttctccgagtgc	30	348
28896	Coding	1116	gtgccgcaaaaggtcttc	66	349
28897	Coding	1125	tactcaggtgtgccgcaa	66	350
28898	Coding	1461	ggcttgaagggtgggctg	41	351
28899	Coding	1497	tcaaaatacctggtgtca	51	352

28900	Coding	1512	gccgtgaactcctcatca	56	353
28901	Coding	1541	ggtcaggtggtgtgatgg	0	354
28902	Coding	1573	ctcgctgtccacacactc	61	355
28903	3' UTR	1671	gcctctccatccctccaa	76	356
28904	3' UTR	1739	acagcgtggcttctctca	12	357
28905	3' UTR	1814	ttttcttccctaccccgc	64	358
28906	3' UTR	1819	gatagttttcttccctac	0	359
28907	3' UTR	1831	taaaacccgcaggatagt	74	360
28908	3' UTR	1856	ggagaacaaactggatga	0	361
28909	3' UTR	1987	ctggctgacagagtgagg	59	362
28910	3' UTR	1991	gcggctggctgacagagt	61	363
28911	3' UTR	2031	cccagagagatgacagat	46	364
28912	3' UTR	2127	gctgctgtgtgcctgcca	38	365
28913	3' UTR	2264	cataatacacaataacaa	39	366
28914	3' UTR	2274	atttgaacaacataatac	11	367
28915	3' UTR	2397	aagtgctaccgtggagag	57	368
28916	3' UTR	2407	cgaaaaggtcaagtgcta	41	369
28917	3' UTR	2453	cagggagtcagggagggc	13	370
28918	3' UTR	2545	aaagttgaatgttgtaaa	10	371
28919	3' UTR	2553	aaaatactaaagttgaat	25	372

EXAMPLE 31: Antisense inhibition of Akt-1 expression- phosphorothioate 2'-MOE gapmer oligonucleotides

In accordance with the present invention, a second series of oligonucleotides targeted to human Akt-1 were synthesized. The oligonucleotide sequences are shown in Table 19. Target sites are indicated by nucleotide numbers, as given in the sequence source reference (Genbank accession no. M63167), to which the oligonucleotide binds.

All compounds in Table 19 are chimeric oligonucleotides ("gapmers") 18 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by four-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. Cytidine residues in the 2'-MOE wings are 5-methylcytidines.

Data were obtained by real-time quantitative PCR as described in other examples herein and are averaged from three experiments. If present, "N.D." indicates "no data".

Table 19

Inhibition of Akt-1 mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS#	REGION	TARGET SITE	SEQUENCE	% Inhibition	SEQ ID NO.
28920	5' UTR	4	ccctgtgccctgtcccag	88	333
28921	5' UTR	27	cctaagcccctggtgaca	44	334
28922	5' UTR	62	ctttgacttctttgaccc	61	335
28923	5' UTR	70	ggcagccccttgacttc	79	336
28924	Coding	213	caaccctccttcacaata	72	337
28925	Coding	234	tactcccctcgtttgtgc	39	338
28926	Coding	281	tgccatcattcttgagga	73	339
28927	Coding	293	agccaatgaaggtgccat	62	340
28928	Coding	352	cacagagaagttgttgag	48	341
28929	Coding	496	agtctggatggcggttgt	43	342
28930	Coding	531	tcctcctcctcctgcttc	49	343
28931	Coding	570	cctgagttgtcactgggt	71	344
28932	Coding	666	ccgaaagtgcccttgccc	64	345
28933	Coding	744	gccacgatgacttccttc	66	346
28934	Coding	927	cggtcctcggagaacaca	77	347
28935	Coding	990	acgttcttctccgagtgc	89	348
28936	Coding	1116	gtgccgcaaaaggtcttc	61	349
28937	Coding	1125	tactcaggtgtgccgcaa	74	350
28938	Coding	1461	ggcttgaagggtgggctg	54	351
28939	Coding	1497	tcaaaatacctggtgtca	78	352
28940	Coding	1512	gccgtgaactcctcatca	88	353
28941	Coding	1541	ggtcaggtggtgtgatgg	71	354
28942	Coding	1573	ctcgctgtccacacactc	83	355
28943	3' UTR	1671	gcctctccatccctccaa	86	356
28944	3' UTR	1739	acagcgtggcttctctca	73	357
28945	3' UTR	1814	ttttcttccctaccccgc	77	358
28946	3' UTR	1819	gatagttttcttccctac	43	359
28947	3' UTR	1831	taaaacccgcaggatagt	64	360
28948	3' UTR	1856	ggagaacaaactggatga	70	361
28949	3' UTR	1987	ctggctgacagagtgagg	90	362
28950	3' UTR	1991	gcggctggctgacagagt	82	363
28951	3' UTR	2031	cccagagagatgacagat	53	364
28952	3' UTR	2127	gctgctgtgtgcctgcca	80	365
28953	3' UTR	2264	cataatacacaataacaa	48	366
28954	3' UTR	2274	atttgaacaacataatac	39	367
28955	3' UTR	2397	aagtgctaccgtggagag	38	368
28956	3' UTR	2407	cgaaaaggtcaagtgcta	83	369

28957	3' UTR	2453	cagggagtcagggagggc	59	370
28958	3' UTR	2545	aaagttgaatgttgtaaa	25	371
28959	3' UTR	2553	aaaatactaaagttgaat	45	372

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What is claimed is:

1. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating a library of virtual compounds *in silico* according to defined criteria, and evaluating *in silico* the binding of said virtual compounds with said target nucleic acid according to defined criteria.

2. A method of defining a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence comprising generating *in silico* a plurality of virtual oligonucleotides according to defined criteria,and evaluating *in silico* the binding of said plurality of virtual oligonucleotides with said target nucleic acid according to defined criteria.

3. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid comprising, generating *in silico* a library of virtual compounds according to defined criteria wherein said virtual compounds modulate the expression of said target nucleic acid sequence, and robotically synthesizing synthetic compounds corresponding to at least some of said virtual compounds.

4. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid comprising generating *in silico* virtual compounds according to defined criteria wherein said virtual compounds modulate the expression of said target nucleic acid sequence, synthesizing synthetic compounds corresponding to at least some of said virtual compounds, and robotically assaying said synthetic compounds for one or more desired physical, chemical or biological properties.

5. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating *in silico* a library of nucleobase sequences according to

defined criteria and evaluating *in silico* a plurality of virtual oligonucleotides having said nucleobase sequences according to defined criteria.

6. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising evaluating *in silico* a plurality of virtual compounds according to defined criteria and robotically synthesizing a plurality of synthetic compounds corresponding to said plurality of virtual compounds.

7. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising evaluating *in silico* a plurality of virtual compounds according to defined criteria and robotically assaying a plurality of synthetic compounds corresponding to at least some of said virtual compounds for one or more desired physical, chemical or biological properties.

8. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating a library of nucleobase sequences *in silico* according to defined criteria and robotically synthesizing a plurality of synthetic compounds compounds having said nucleobase sequences.

9. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising robotically synthesizing a plurality of synthetic compounds and robotically assaying said plurality of synthetic compounds for one or more desired physical, chemical or biological properties.

10. A method of defining a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising generating a library of nucleobase sequences *in silico* according to

defined criteria and robotically assaying a plurality of synthetic compounds having at least some of said nucleobase sequences for one or more desired physical, chemical or biological properties.

11. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides.

12. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically assaying a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides for one or more desired physical, chemical or biological properties.

13. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucletoides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides having at least

some of said nucleobase sequences; and

(c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

14. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

(c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

15. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria;

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

(d) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

16. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) choosing an oligonucleotide chemistry;

(c) robotically synthesizing a set of synthetic oligonucleotides having said nucleobase sequences of step (a) and said oligonucleotide chemistry of step (b);

(d) robotically assaying said set of synthetic oligonucleotides of step (c) for a physical, chemical or biological activity; and

(e) selecting a subset of said set of synthetic oligonucleotides of step (c) having a desired level of physical, chemical or biological activity in order to generate said set of compounds.

17. A method of generating a set of oligonucleotides that modulate the expression of a target nucleic acid sequence via binding of said oligonucleotides with said target nucleic acid sequence, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) choosing an oligonucleotide chemistry;

(c) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) and the oligonucleotide chemistry of (b) according to defined criteria, and selecting those having preferred characteristics, in order to generate a set of preferred nucleobase sequences;

(d) robotically synthesizing a set of synthetic oligonucleotides having said preferred nucleobase sequences of step (c) and said oligonucleotide chemistry of step (b);

(e) robotically assaying said set of synthetic oligonucleotides of step (d) for a physical, chemical or biological activity; and

(f) selecting a subset of said set of synthetic oligonucleotides of step (d) having a desired level of physical, chemical or biological activity in order to generate said set of oligonucleotides.

18. The method of claim 12, wherein said step of robotically assaying said plurality of synthetic oligonucleotide compounds is performed by computer-controlled real-time polymerase chain reaction or by computer-controlled enzyme-linked immunosorbent assay.

19. The method of claim 11, wherein said target nucleic acid sequence is that of a genomic DNA, a cDNA, a product of a polymerase chain reaction, an expressed sequence tag, an mRNA or a structural RNA.

20. The method of claim 11, wherein said target nucleic acid sequence is a human nucleic acid.

21. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating a library of antisense nucleobase sequences *in silico* according to defined criteria.

22. A method of identifying a set of compounds that modulate the expression of a target nucleic acid sequence via binding of said compounds with said target nucleic acid sequence comprising evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria.

23. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an compound to said nucleic acid sequences comprising robotically synthesizing a plurality of synthetic antisense compounds.

24. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an compound to said nucleic acid sequences comprising robotically assaying a plurality of synthetic antisense compounds for one or more desired physical, chemical or biological properties.

25. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating *in silico* a library of nucleobase sequences according to defined criteria and evaluating *in silico* a plurality of virtual oligonucleotides having said nucleobase sequences according to defined criteria.

26. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria and robotically synthesizing a plurality of synthetic oligonucleotides corresponding to least some of said virtual oligonucleotides.

27. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria and robotically assaying a plurality of synthetic oligonucleotides corresponding to least some of said virtual oligonucleotides for one or more desired physical, chemical or biological properties.

28. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating a library of nucleobase sequences *in silico* according to defined criteria and robotically synthesizing a plurality of synthetic oligonucleotides having said nucleobase sequences.

29. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising robotically synthesizing a plurality of synthetic oligonucleotides and robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

30. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences comprising generating a library of nucleobase sequences *in silico* according to defined criteria and robotically assaying a plurality of synthetic oligonucleotides having said nucleobase sequences for one or more desired physical, chemical or biological properties.

31. A method of identifying one or more nucleic acid sequences amenable to antisense

binding of an oligonucleotide to said nucleic acid sequences comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides.

32. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically assaying a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides for one or more desired physical, chemical or biological properties.

33. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides having at least some of said nucleobase sequences; and

(c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

34. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

(c) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

35. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria;

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to least some of said plurality of virtual oligonucleotides; and

(d) robotically assaying said plurality of synthetic oligonucleotides for one or more desired physical, chemical or biological properties.

36. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) choosing an oligonucleotide chemistry;

(c) robotically synthesizing a set of synthetic oligonucleotides having said nucleobase sequences of step (a) and said oligonucleotide chemistry of step (b);

(d) robotically assaying said set of synthetic oligonucleotides of step (c) for a physical, chemical or biological activity; and

(e) selecting a subset of said set of synthetic oligonucleotides of step (c) having a desired level of physical, chemical or biological activity.

37. A method of identifying one or more nucleic acid sequences amenable to antisense binding of an oligonucleotide to said nucleic acid sequences, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined

criteria;

(b) choosing an oligonucleotide chemistry;

(c) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria, and selecting those having preferred characteristics, in order to generate a set of preferred nucleobase sequences;

(d) robotically synthesizing a set of synthetic oligonucleotides having said preferred nucleobase sequences of step (b) and said oligonucleotide chemistry of step (c);

(e) robotically assaying said set of synthetic oligonucleotides of step (d) for a physical, chemical or biological activity; and

(f) selecting a subset of said set of oligonucleotides of step (d) having a desired level of physical, chemical or biological activity.

38. The method of claim 32, wherein said step of robotically assaying said plurality of synthetic antisense oligonucleotides is performed by computer-controlled real-time polymerase chain reaction or by computer-controlled enzyme-linked immunosorbent assay.

39. The method of claim 31, wherein said nucleic acid sequence is that of a genomic DNA, a cDNA, a product of a polymerase chain reaction, an expressed sequence tag, an mRNA or a structural RNA.

40. The method of claim 31, wherein said nucleic acid sequence is a human nucleic acid.

41. A computer formatted medium comprising computer readable instructions for identifying compounds that have one or more desired properties according to defined criteria and that bind to a genomic DNA, a cDNA, a product of a polymerase chain reaction, an expressed sequence tag, an mRNA or a structural RNA.

42. A computer formatted medium comprising computer readable instructions for performing the method of any one of claims 1 to 20.

43. A computer formatted medium comprising computer readable instructions for performing a method of identifying one or more nucleic acid sequences amenable to antisense binding of a compound to said nucleic acid sequences.

44. A computer formatted medium comprising computer readable instructions for performing the method of any one of claims 21 to 40.

45. A computer formatted medium comprising one or more nucleic acid sequences amenable to antisense binding of a compound to said nucleic acid sequences in computer readable form.

46. A computer formatted medium comprising one or more nucleic acid sequences amenable to antisense binding of a compound to said nucleic acid sequences in computer readable form, wherein said one or more nucleic acid sequences is identified according to the method of any one of claims 21-40.

47. A process for validating the function of a gene or the product of said gene comprising generating *in silico* a library of nucleobase sequences targeted to said gene and robotically assaying a plurality of synthetic compounds having at least some of said nucleobase sequences for effects on biological function.

48. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides.

49. A process for validating the function of a gene or the product of said gene,

comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) according to defined criteria; and

(c) robotically assaying a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides for effects on biological function.

50. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides having at least some of said nucleobase sequences; and

(c) robotically assaying said plurality of synthetic oligonucleotides for effects on biological function.

51. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) evaluating *in silico* a plurality of virtual oligonucleotides according to defined criteria;

(b) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

(c) robotically assaying said plurality of synthetic oligonucleotides for effects on biological function.

52. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase

sequences of (a) according to defined criteria;

(c) robotically synthesizing a plurality of synthetic oligonucleotides corresponding to at least some of said virtual oligonucleotides; and

(d) robotically assaying said plurality of synthetic oligonucleotides for effects on biological function.

53. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) choosing an oligonucleotide chemistry;

(c) robotically synthesizing a set of synthetic oligonucleotides having said nucleobase sequences of step (a) and said oligonucleotide chemistry of step (b);

(d) robotically assaying said set of synthetic oligonucleotides of step (c) for effects on biological function; and

(e) selecting a subset of said set of synthetic oligonucleotides of step (c) having a desired level of physical, chemical or biological activity in order to generate said set of compounds.

54. A process for validating the function of a gene or the product of said gene, comprising the steps of:

(a) generating a library of nucleobase sequences *in silico* according to defined criteria;

(b) choosing an oligonucleotide chemistry;

(c) evaluating *in silico* a plurality of virtual oligonucleotides having the nucleobase sequences of (a) and the oligonucleotide chemistry of (b) according to defined criteria, and selecting those having preferred characteristics, in order to generate a set of preferred nucleobase sequences;

(d) robotically synthesizing a set of synthetic oligonucleotides having said preferred nucleobase sequences of step (c) and said oligonucleotide chemistry of step (b);

(e) robotically assaying said set of synthetic oligonucleotides of step (d) for effects

on biological function; and

(f) selecting a subset of said set of synthetic oligonucleotides of step (d) having a desired level of physical, chemical or biological activity in order to generate said set of oligonucleotides.

5

ABSTRACT

Iterative, preferably computer based iterative processes for generating synthetic compounds with desired physical, chemical and/or bioactive properties, i.e., active compounds, are provided. During iterations of the processes, a target nucleic acid sequence is provided or selected, and a library of candidate nucleobase sequences is generated *in silico* according to defined criteria. A "virtual" oligonucleotide chemistry is chosen and a library of virtual oligonucleotide compounds having the selected nucleobase sequences is generated. These virtual compounds are reviewed and compounds predicted to have particular properties are selected. The selected compounds are robotically synthesized and are preferably robotically assayed for a desired physical, chemical or biological activity. Active compounds are thus generated and, at the same time, preferred sequences and regions of the target nucleic acid that are amenable to oligonucleotide or sequence-based modulation are identified.

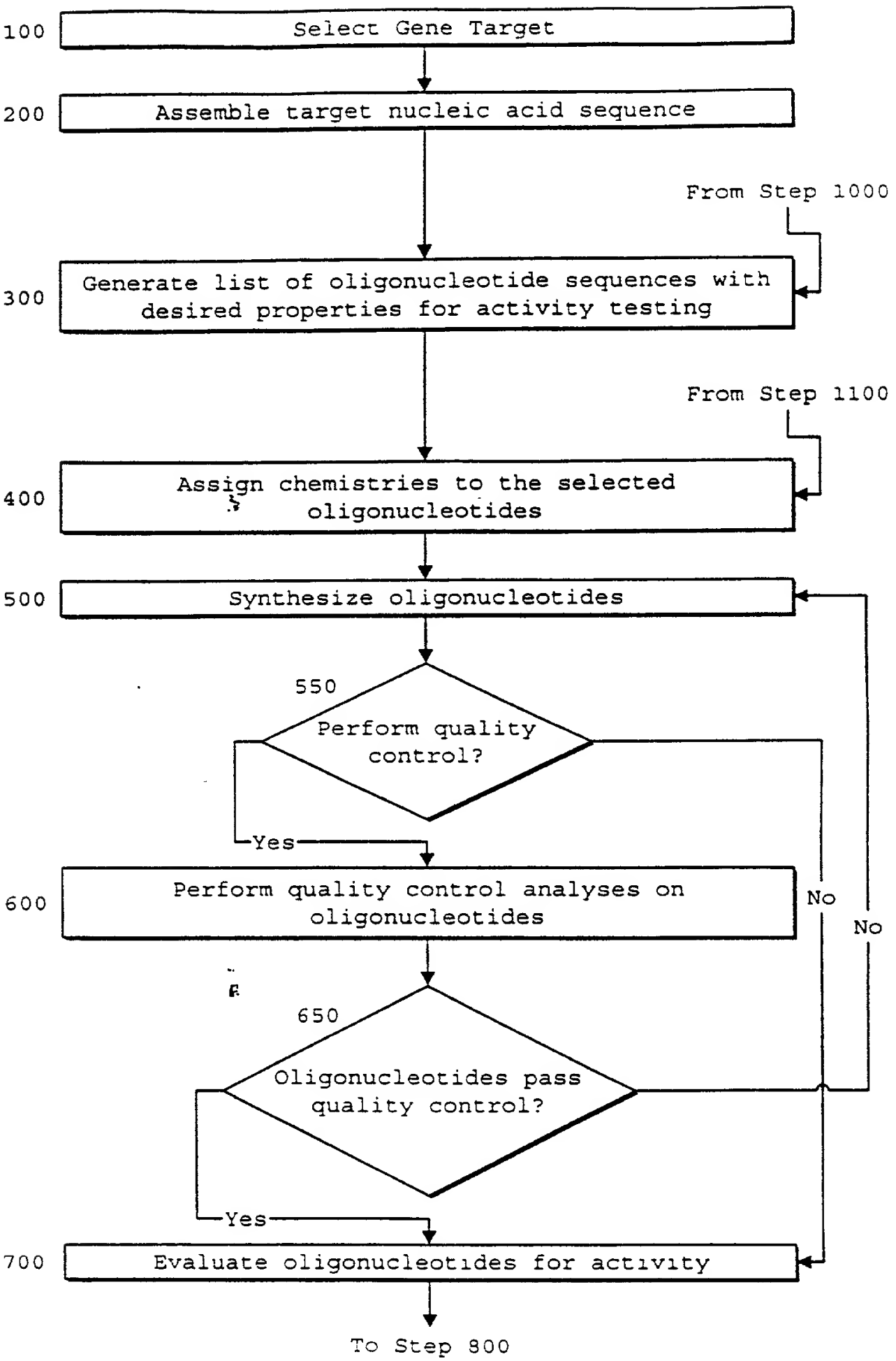

100
Select Gene Target
200
Assemble target nucleic acid sequence
From Step 1000
300
Generate list of oligonucleotide sequences with desired properties for activity testing
From Step 1100
400
Assign chemistries to the selected oligonucleotides
500
Synthesize oligonucleotides
550
Perform quality control?
Yes
No
600
Perform quality control analyses on oligonucleotides
650
Oligonucleotides pass quality control?
Yes
No
700
Evaluate oligonucleotides for activity
To Step 800

Figure 1

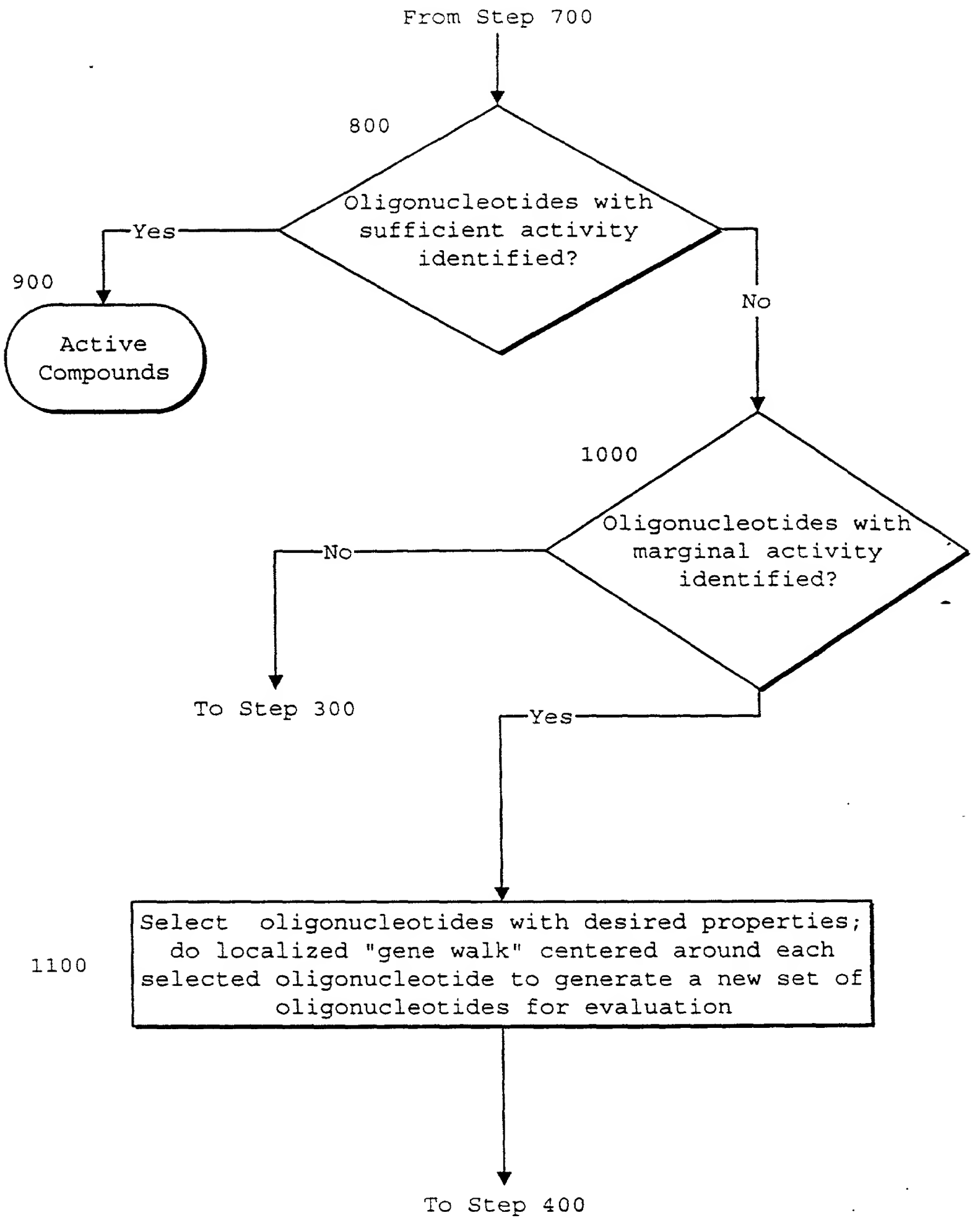

From Step 700
800
Oligonucleotides with sufficient activity identified?
Yes
900
Active Compounds
No
1000
Oligonucleotides with marginal activity identified?
No
To Step 300
Yes
1100
Select oligonucleotides with desired properties; do localized "gene walk" centered around each selected oligonucleotide to generate a new set of oligonucleotides for evaluation
To Step 400

Figure 2

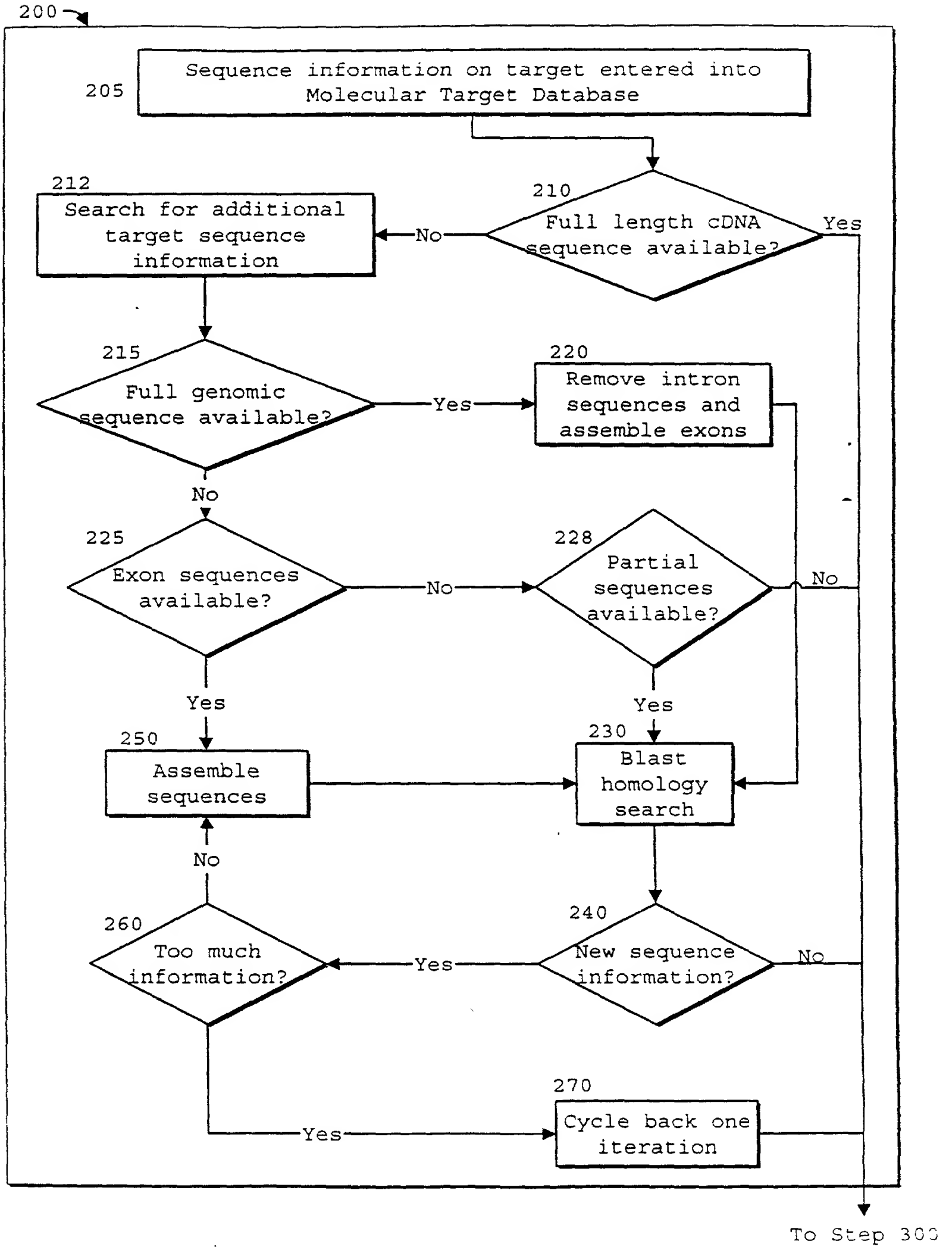

200
205
Sequence information on target entered into Molecular Target Database
210
Full length cDNA sequence available?
No
Yes
212
Search for additional target sequence information
215
Full genomic sequence available?
Yes
220
Remove intron sequences and assemble exons
No
225
Exon sequences available?
No
228
Partial sequences available?
No
Yes
Yes
250
Assemble sequences
230
Blast homology search
No
260
Too much information?
Yes
240
New sequence information?
No
270
Cycle back one iteration
Yes
To Step 300

Figure 3

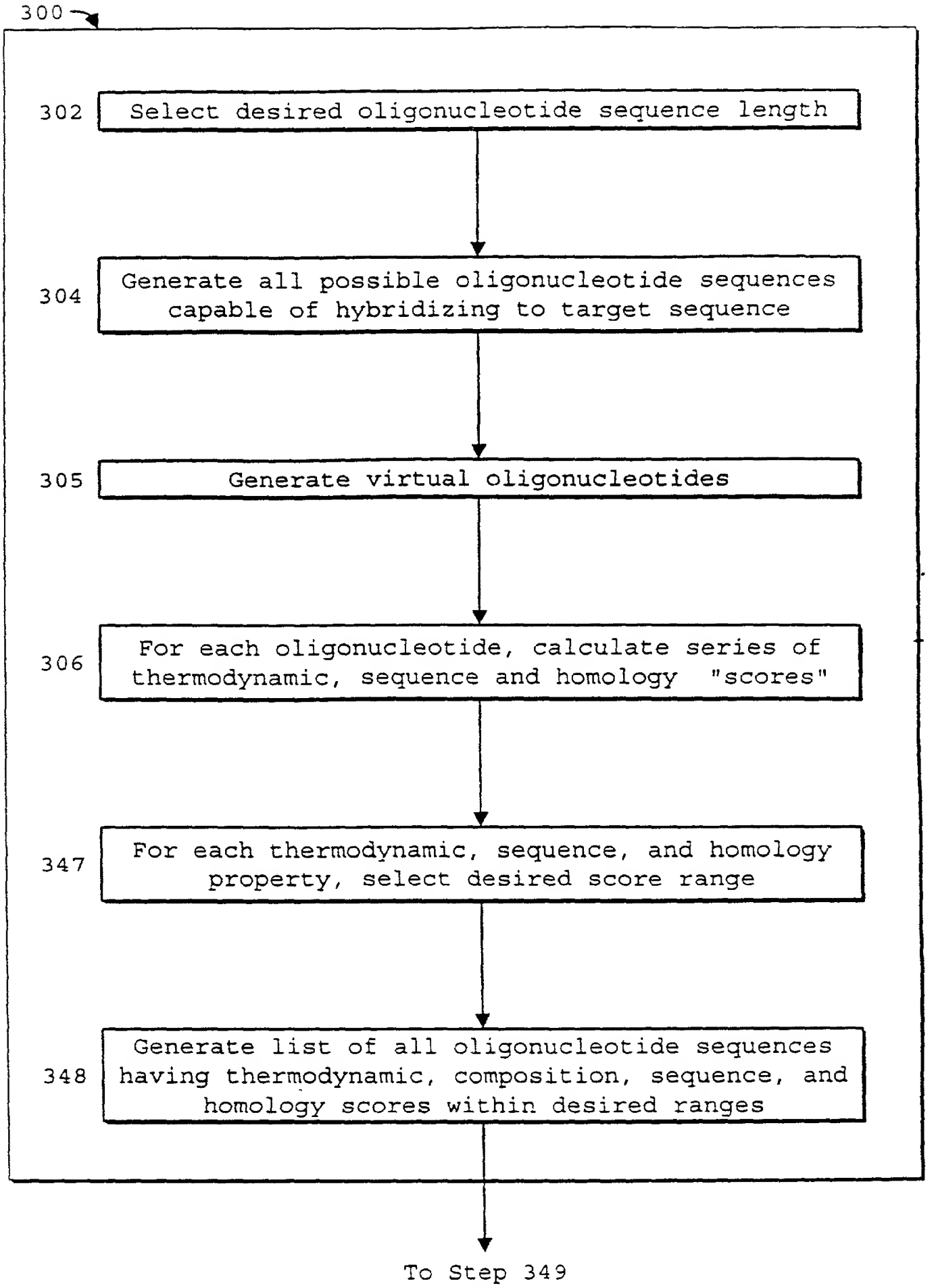

300
302
Select desired oligonucleotide sequence length
304
Generate all possible oligonucleotide sequences capable of hybridizing to target sequence
305
Generate virtual oligonucleotides
306
For each oligonucleotide, calculate series of thermodynamic, sequence and homology "scores"
347
For each thermodynamic, sequence, and homology property, select desired score range
348
Generate list of all oligonucleotide sequences having thermodynamic, composition, sequence, and homology scores within desired ranges
To Step 349

Figure 4

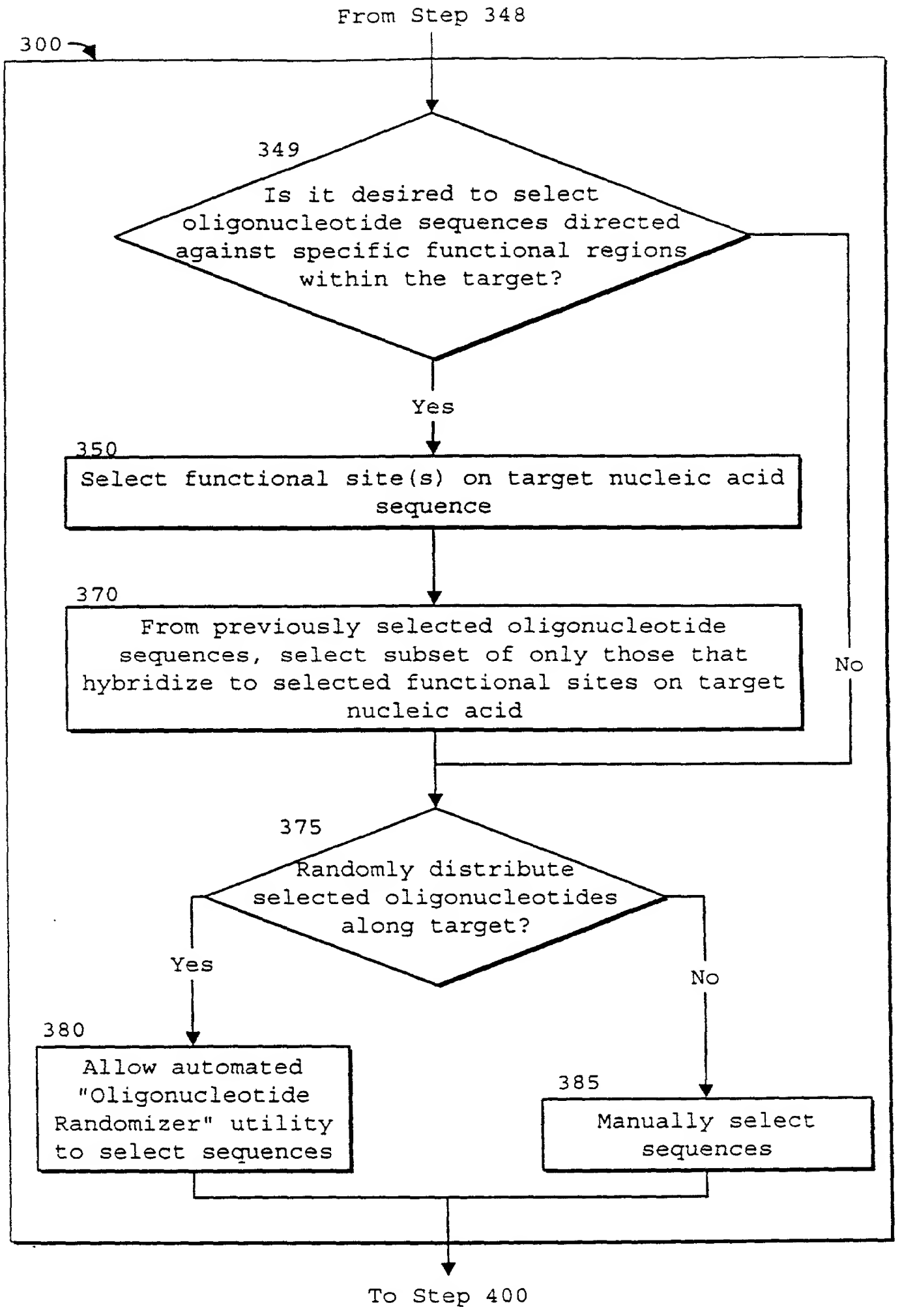

From Step 348
300
349
Is it desired to select oligonucleotide sequences directed against specific functional regions within the target?
Yes
No
350
Select functional site(s) on target nucleic acid sequence
370
From previously selected oligonucleotide sequences, select subset of only those that hybridize to selected functional sites on target nucleic acid
375
Randomly distribute selected oligonucleotides along target?
Yes
No
380
Allow automated "Oligonucleotide Randomizer" utility to select sequences
385
Manually select sequences
To Step 400

Figure 5

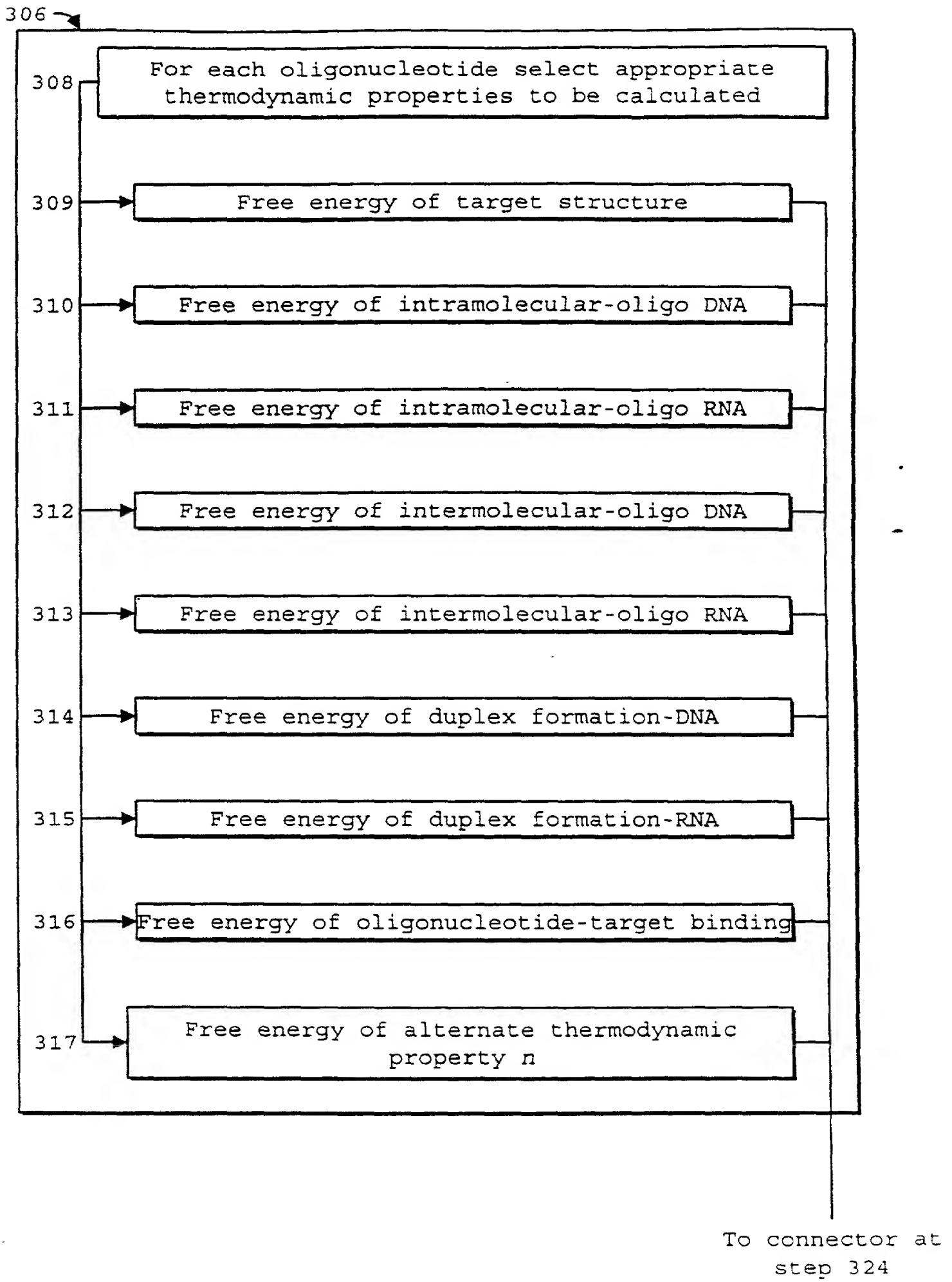

306
308
For each oligonucleotide select appropriate thermodynamic properties to be calculated
309
Free energy of target structure
310
Free energy of intramolecular-oligo DNA
311
Free energy of intramolecular-oligo RNA
312
Free energy of intermolecular-oligo DNA
313
Free energy of intermolecular-oligo RNA
314
Free energy of duplex formation-DNA
315
Free energy of duplex formation-RNA
316
Free energy of oligonucleotide-target binding
317
Free energy of alternate thermodynamic property n
To connector at step 324

Figure 6

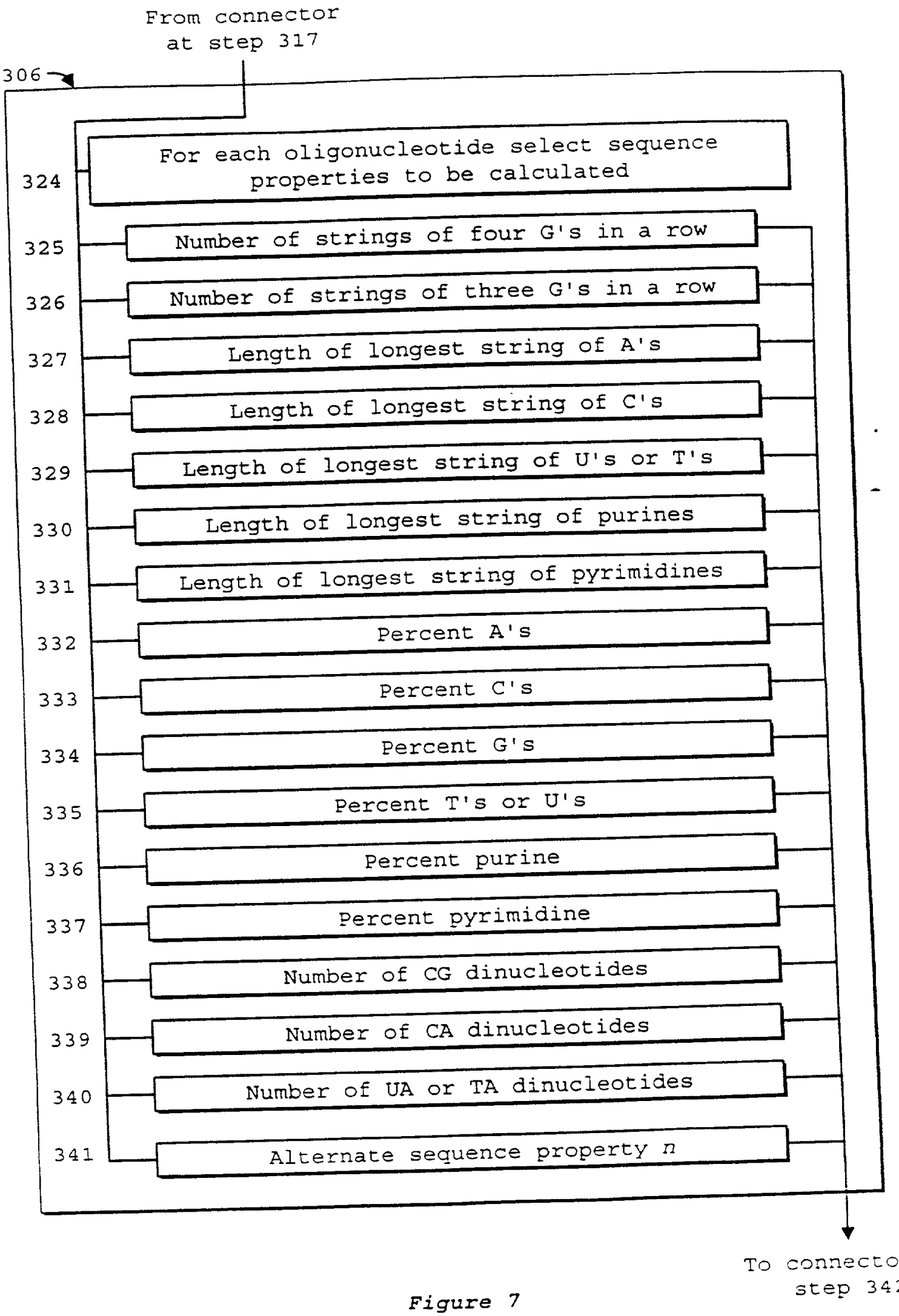

From connector at step 317
306
324
For each oligonucleotide select sequence properties to be calculated
325
Number of strings of four G's in a row
326
Number of strings of three G's in a row
327
Length of longest string of A's
328
Length of longest string of C's
329
Length of longest string of U's or T's
330
Length of longest string of purines
331
Length of longest string of pyrimidines
332
Percent A's
333
Percent C's
334
Percent G's
335
Percent T's or U's
336
Percent purine
337
Percent pyrimidine
338
Number of CG dinucleotides
339
Number of CA dinucleotides
340
Number of UA or TA dinucleotides
341
Alternate sequence property n
To connector at step 342

Figure 7

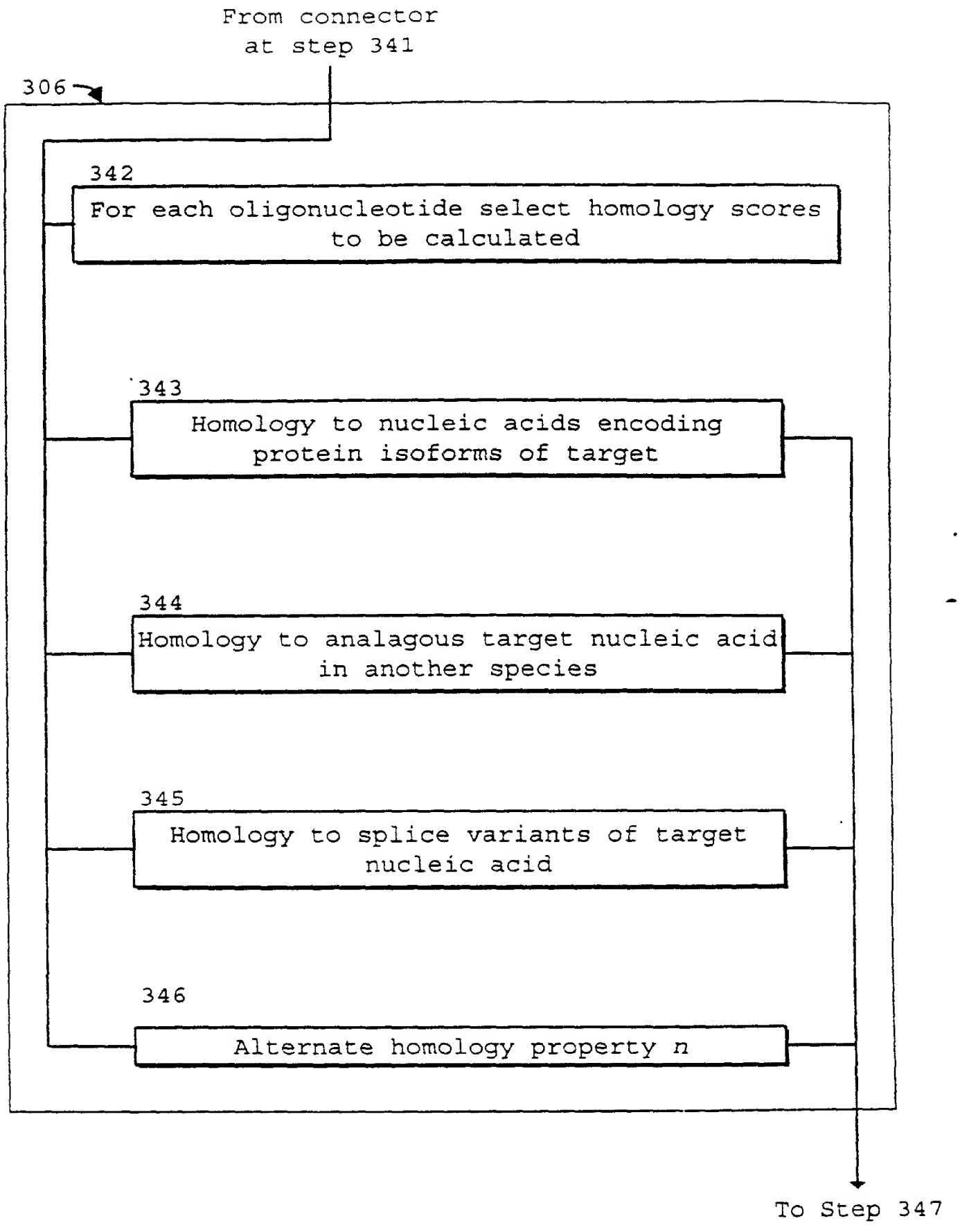

From connector
at step 341
306
342
For each oligonucleotide select homology scores to be calculated
343
Homology to nucleic acids encoding protein isoforms of target
344
Homology to analagous target nucleic acid in another species
345
Homology to splice variants of target nucleic acid
346
Alternate homology property n
To Step 347

Figure 8

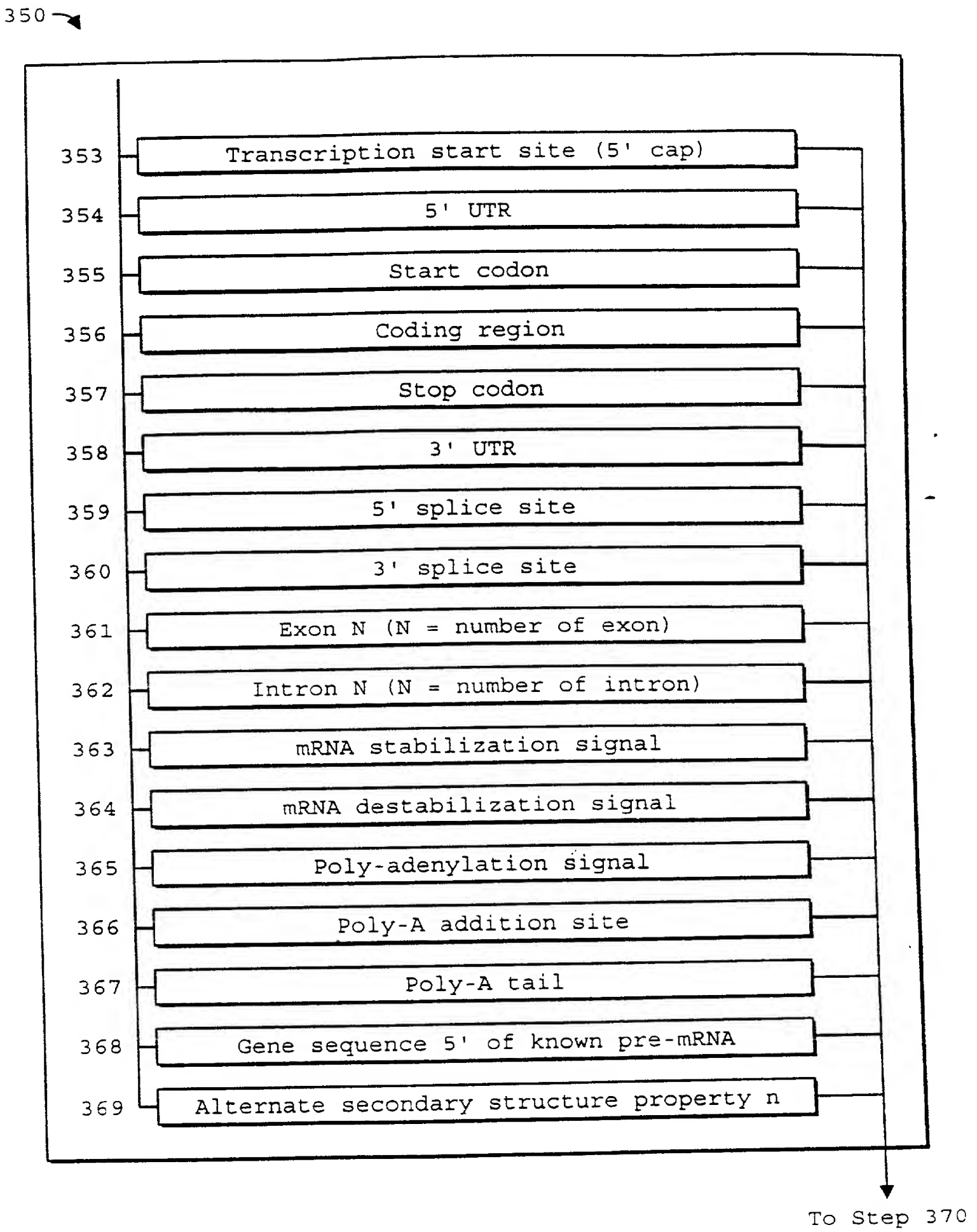

350
353
Transcription start site (5' cap)
354
5' UTR
355
Start codon
356
Coding region
357
Stop codon
358
3' UTR
359
5' splice site
360
3' splice site
361
Exon N (N = number of exon)
362
Intron N (N = number of intron)
363
mRNA stabilization signal
364
mRNA destabilization signal
365
Poly-adenylation signal
366
Poly-A addition site
367
Poly-A tail
368
Gene sequence 5' of known pre-mRNA
369
Alternate secondary structure property n
To Step 370

Figure 9

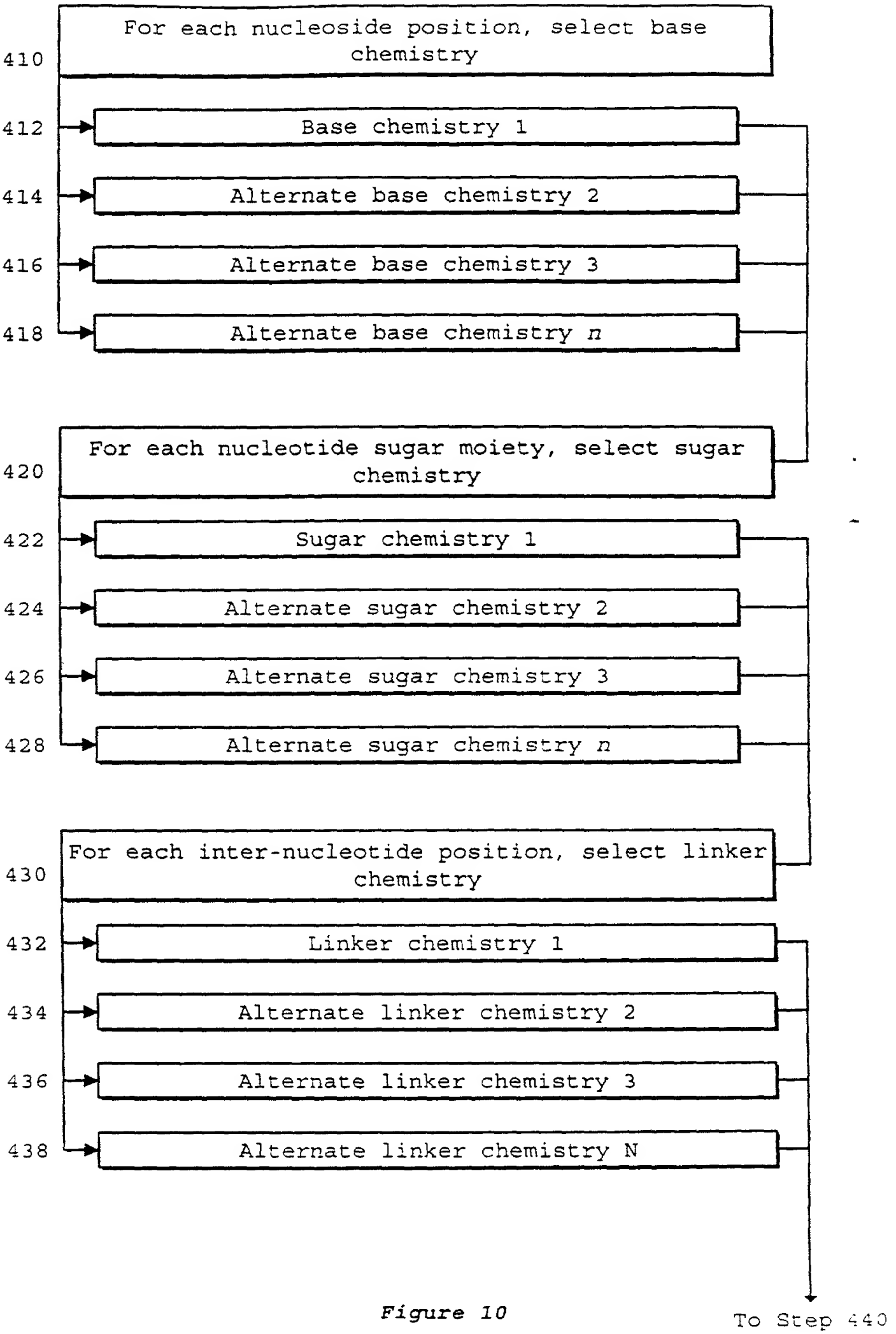

For each nucleoside position, select base chemistry
410
Base chemistry 1
412
Alternate base chemistry 2
414
Alternate base chemistry 3
416
Alternate base chemistry n
418
For each nucleotide sugar moiety, select sugar chemistry
420
Sugar chemistry 1
422
Alternate sugar chemistry 2
424
Alternate sugar chemistry 3
426
Alternate sugar chemistry n
428
For each inter-nucleotide position, select linker chemistry
430
Linker chemistry 1
432
Alternate linker chemistry 2
434
Alternate linker chemistry 3
436
Alternate linker chemistry N
438
To Step 440

Figure 10

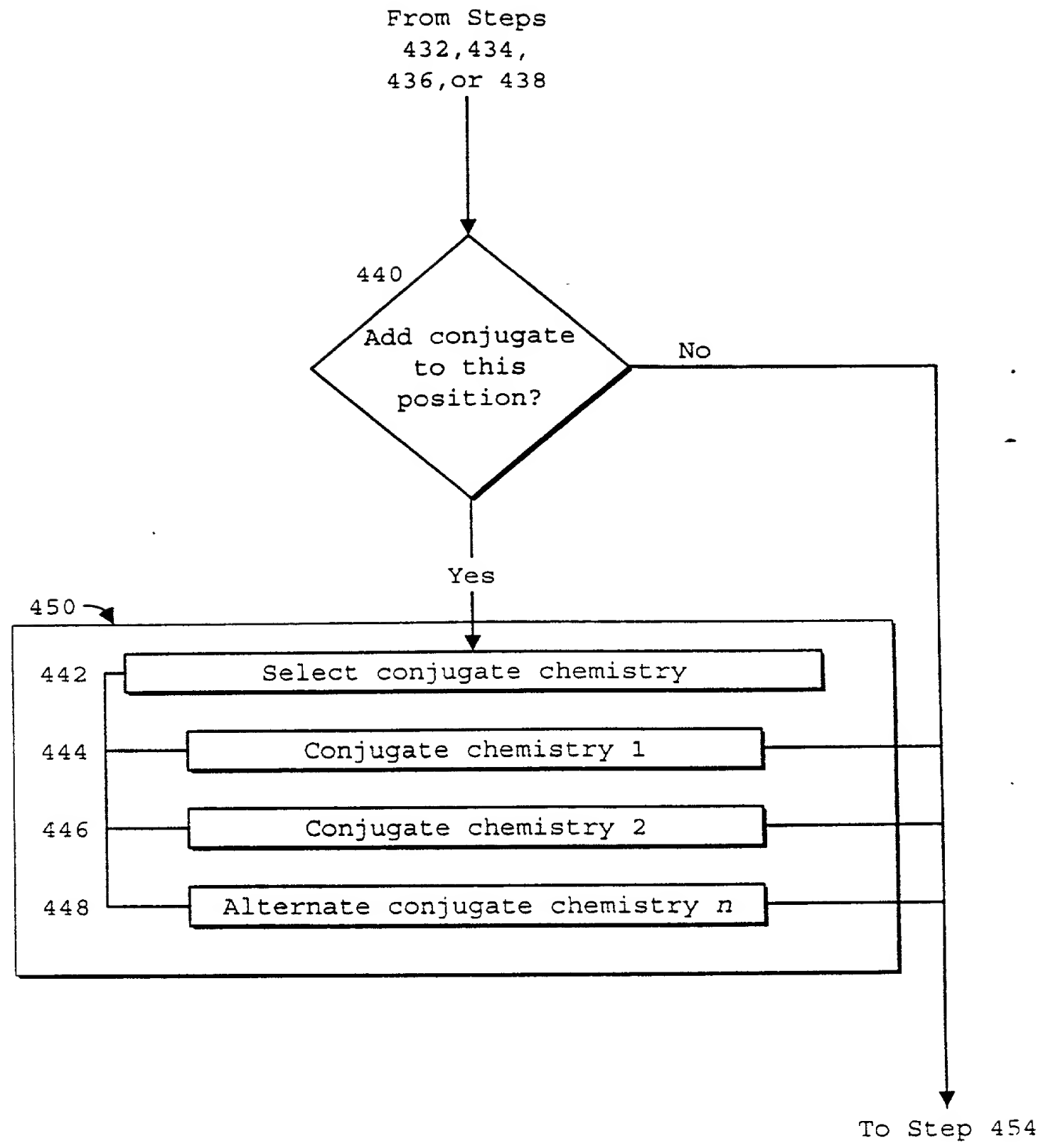

From Steps
432,434,
436,or 438
440
Add conjugate
to this
position?
No
Yes
450
442
Select conjugate chemistry
444
Conjugate chemistry 1
446
Conjugate chemistry 2
448
Alternate conjugate chemistry n
To Step 454

Figure 11

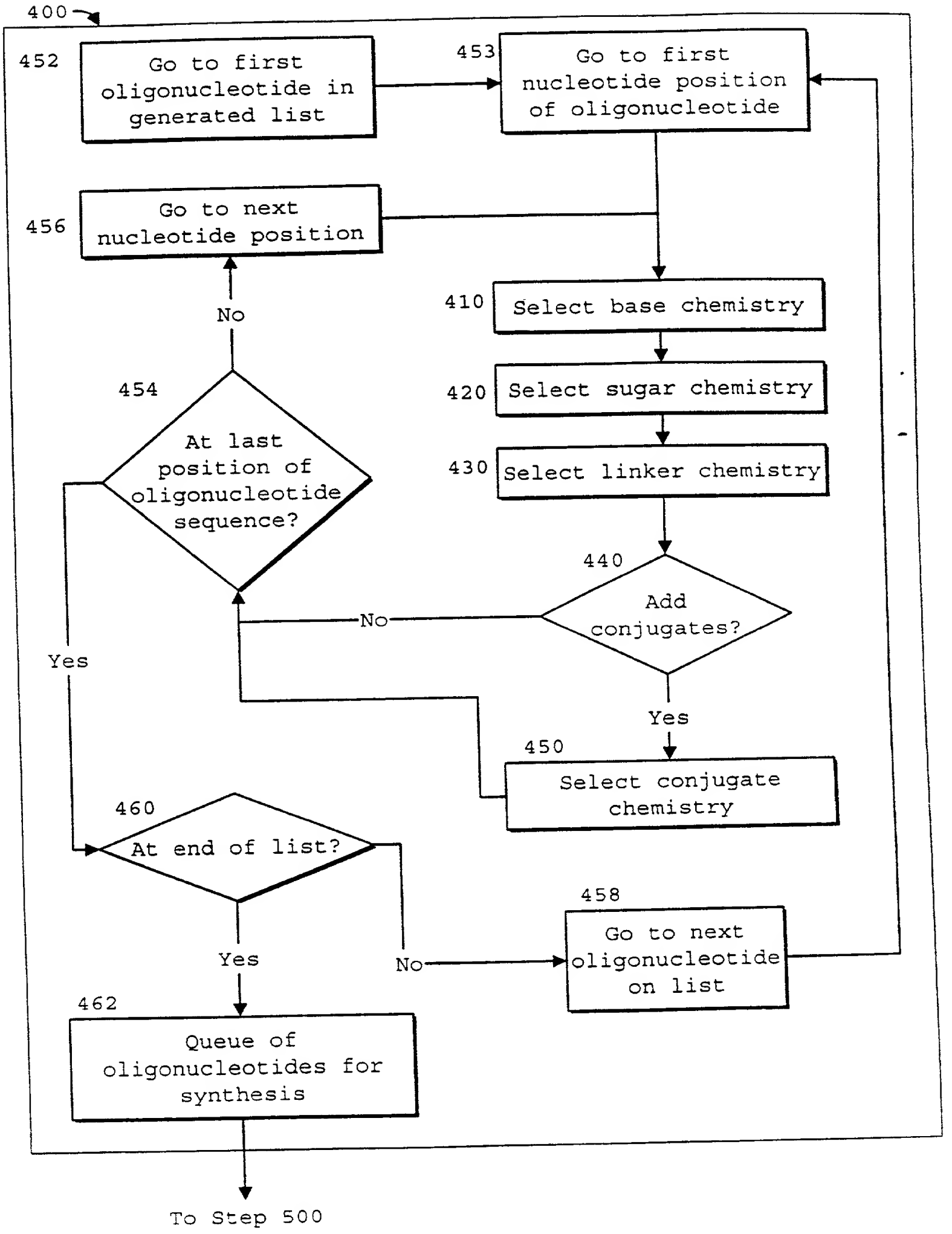

400
452
Go to first oligonucleotide in generated list
453
Go to first nucleotide position of oligonucleotide
456
Go to next nucleotide position
No
410
Select base chemistry
420
Select sugar chemistry
430
Select linker chemistry
454
At last position of oligonucleotide sequence?
440
Add conjugates?
No
Yes
Yes
450
Select conjugate chemistry
460
At end of list?
458
Go to next oligonucleotide on list
Yes
No
462
Queue of oligonucleotides for synthesis
To Step 500

Figure 12

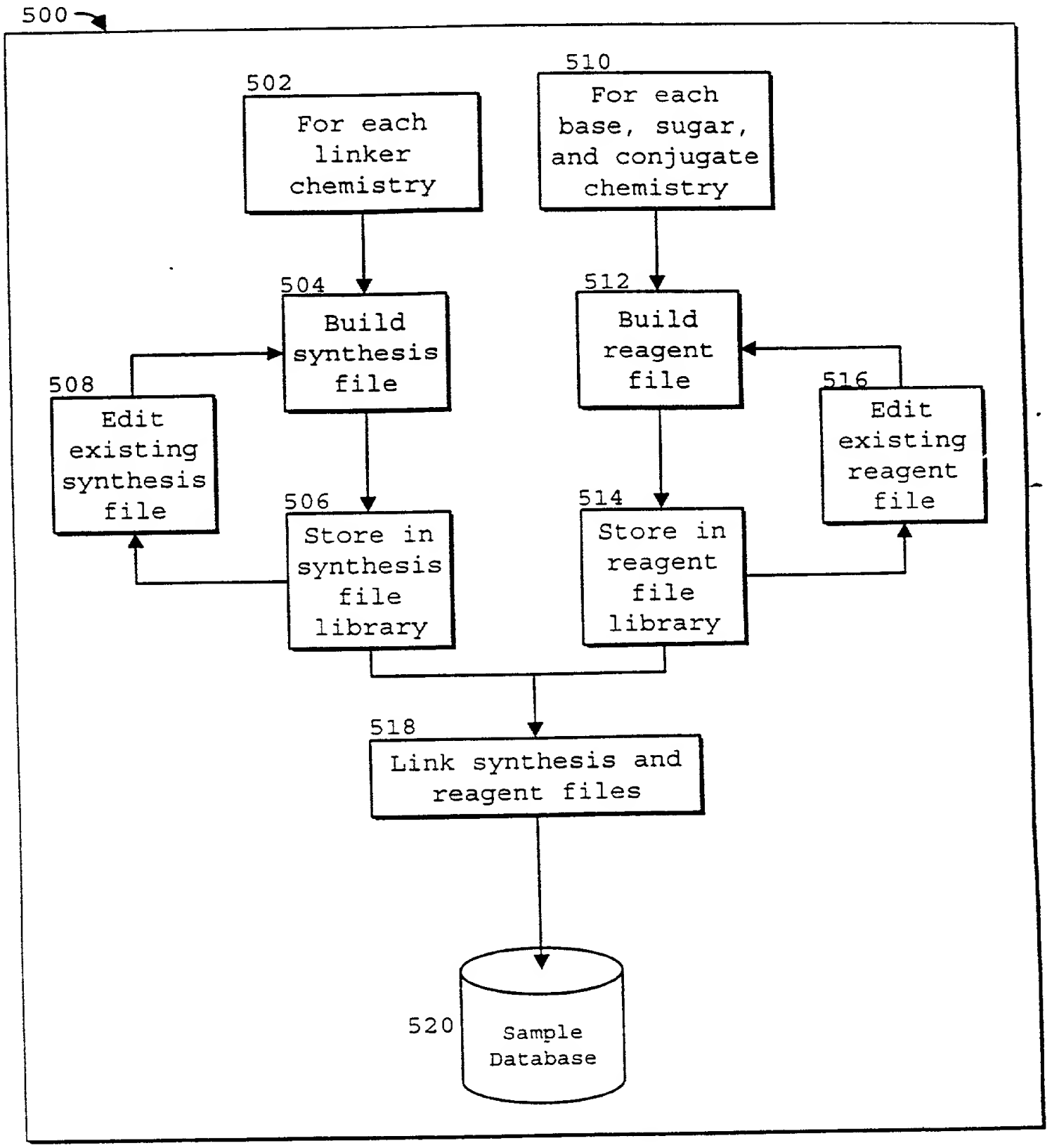

500
502
For each linker chemistry
504
Build synthesis file
508
Edit existing synthesis file
506
Store in synthesis file library
510
For each base, sugar, and conjugate chemistry
512
Build reagent file
516
Edit existing reagent file
514
Store in reagent file library
518
Link synthesis and reagent files
520
Sample Database

Figure 13

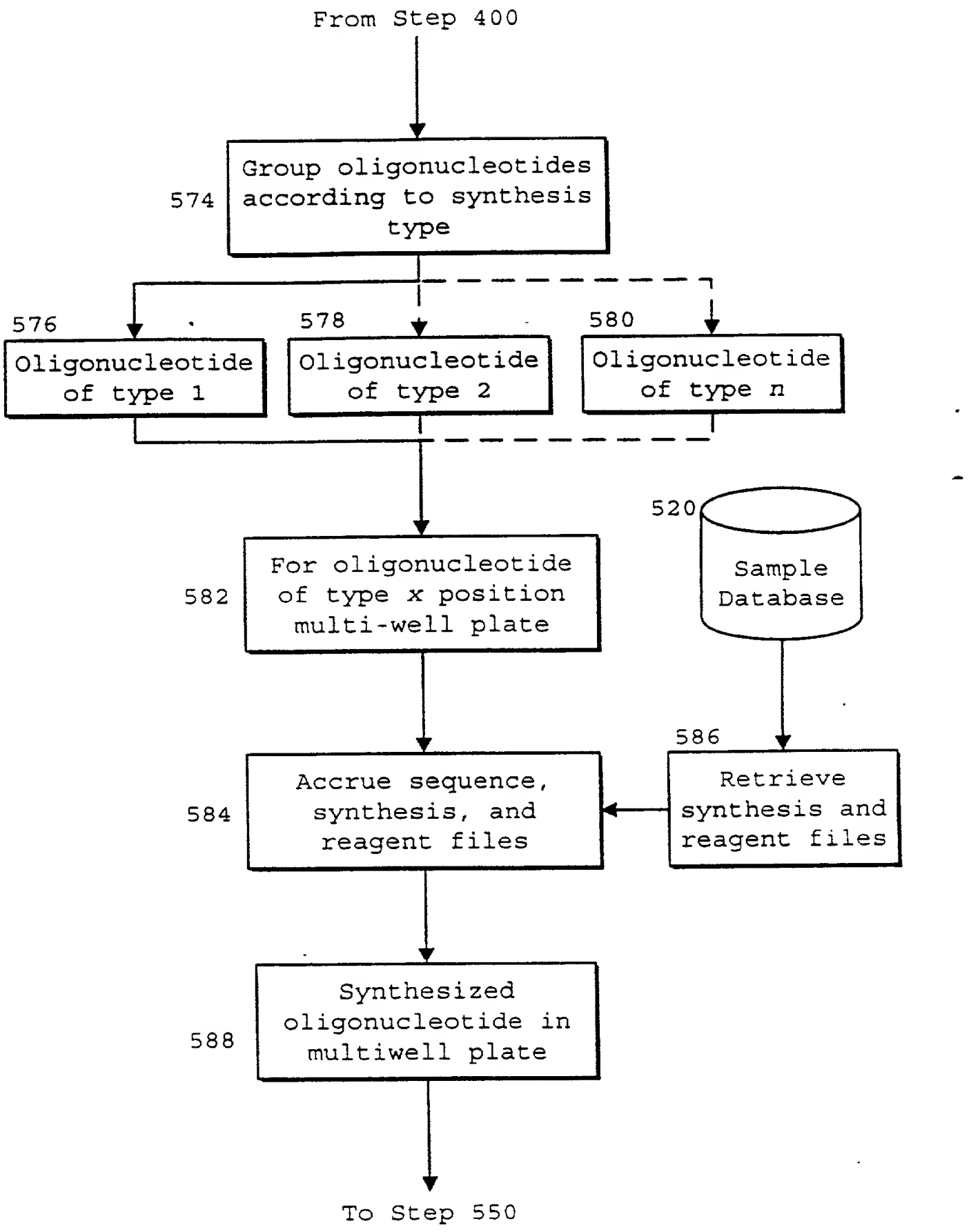

From Step 400
574
Group oligonucleotides according to synthesis type
576
Oligonucleotide of type 1
578
Oligonucleotide of type 2
580
Oligonucleotide of type n
582
For oligonucleotide of type x position multi-well plate
520
Sample Database
586
Retrieve synthesis and reagent files
584
Accrue sequence, synthesis, and reagent files
588
Synthesized oligonucleotide in multiwell plate
To Step 550

Figure 14

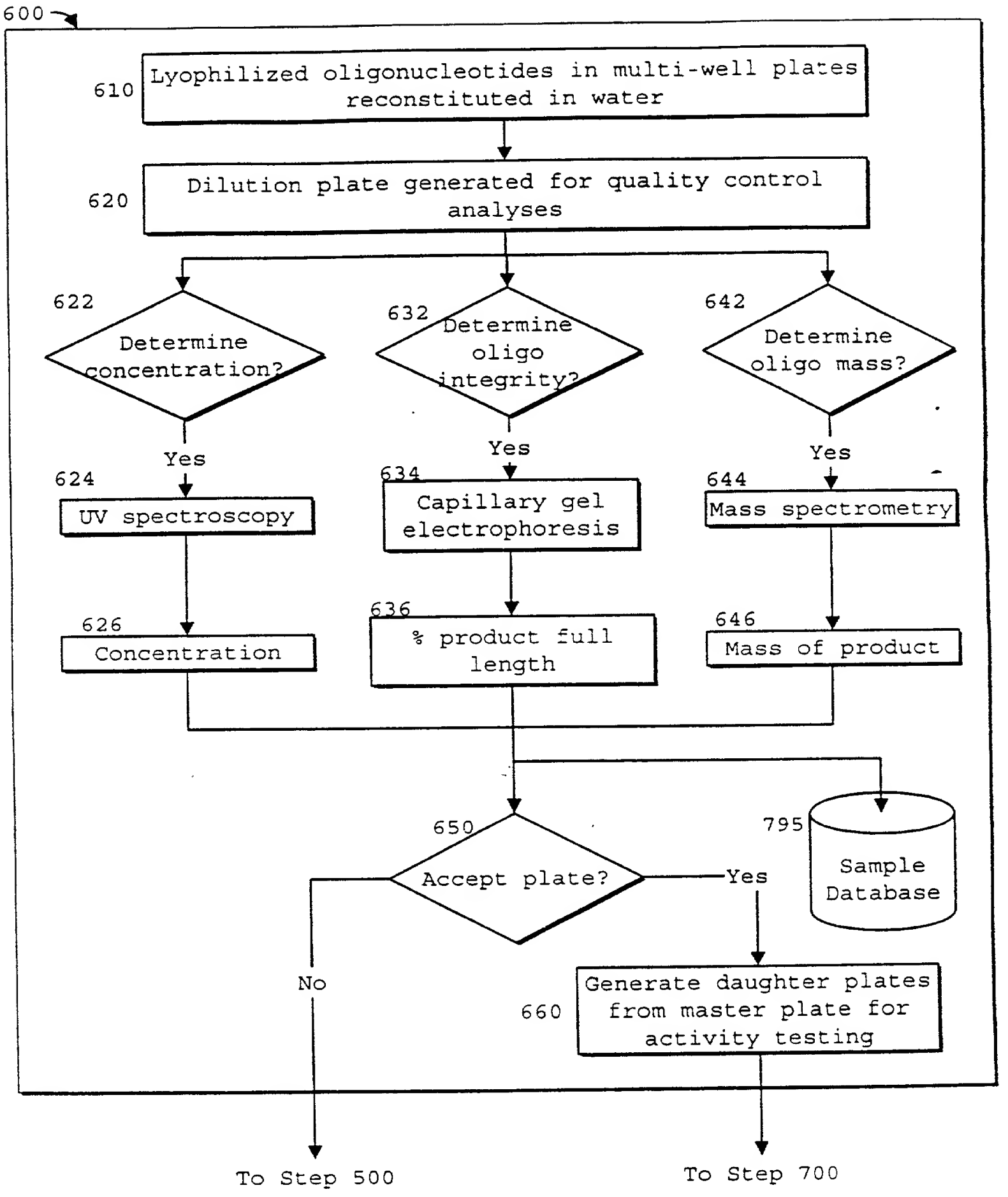

600
610
Lyophilized oligonucleotides in multi-well plates reconstituted in water
620
Dilution plate generated for quality control analyses
622
Determine concentration?
632
Determine oligo integrity?
642
Determine oligo mass?
Yes
624
UV spectroscopy
Yes
634
Capillary gel electrophoresis
Yes
644
Mass spectrometry
626
Concentration
636
% product full length
646
Mass of product
650
Accept plate?
Yes
795
Sample Database
No
660
Generate daughter plates from master plate for activity testing
To Step 500
To Step 700

Figure 15

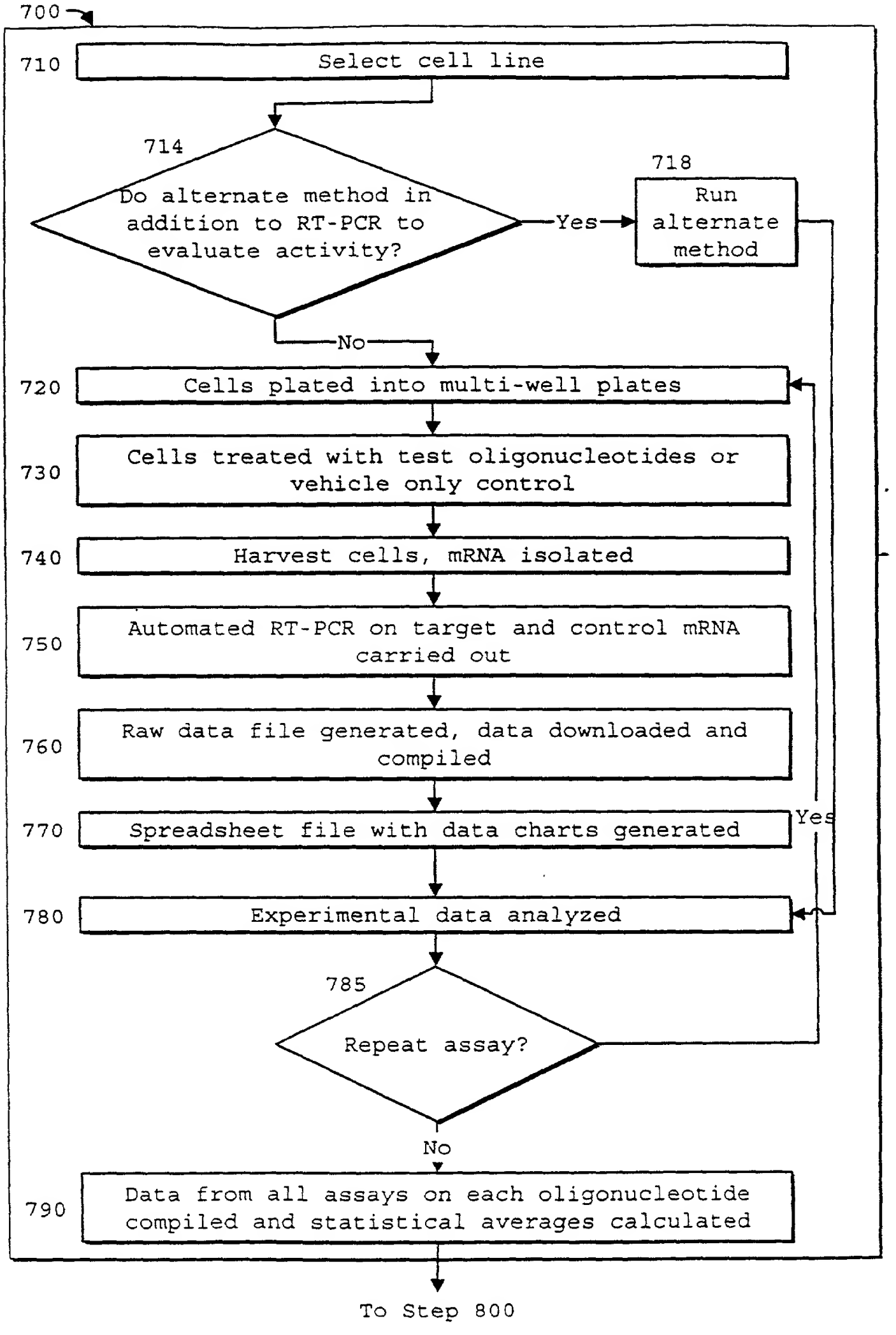

700
710
Select cell line
714
Do alternate method in addition to RT-PCR to evaluate activity?
Yes
718
Run alternate method
No
720
Cells plated into multi-well plates
730
Cells treated with test oligonucleotides or vehicle only control
740
Harvest cells, mRNA isolated
750
Automated RT-PCR on target and control mRNA carried out
760
Raw data file generated, data downloaded and compiled
770
Spreadsheet file with data charts generated
Yes
780
Experimental data analyzed
785
Repeat assay?
No
790
Data from all assays on each oligonucleotide compiled and statistical averages calculated
To Step 800

Figure 16

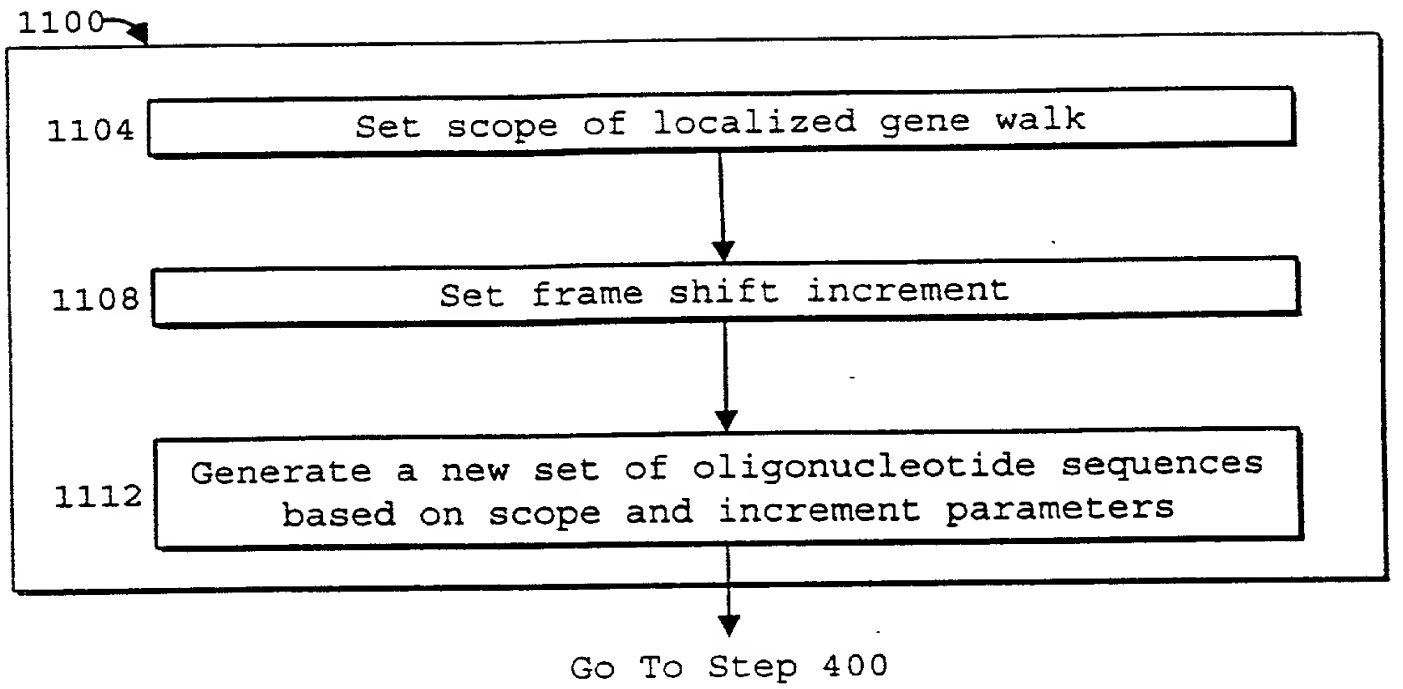

1100
1104
Set scope of localized gene walk
1108
Set frame shift increment
1112
Generate a new set of oligonucleotide sequences based on scope and increment parameters
Go To Step 400

Figure 17

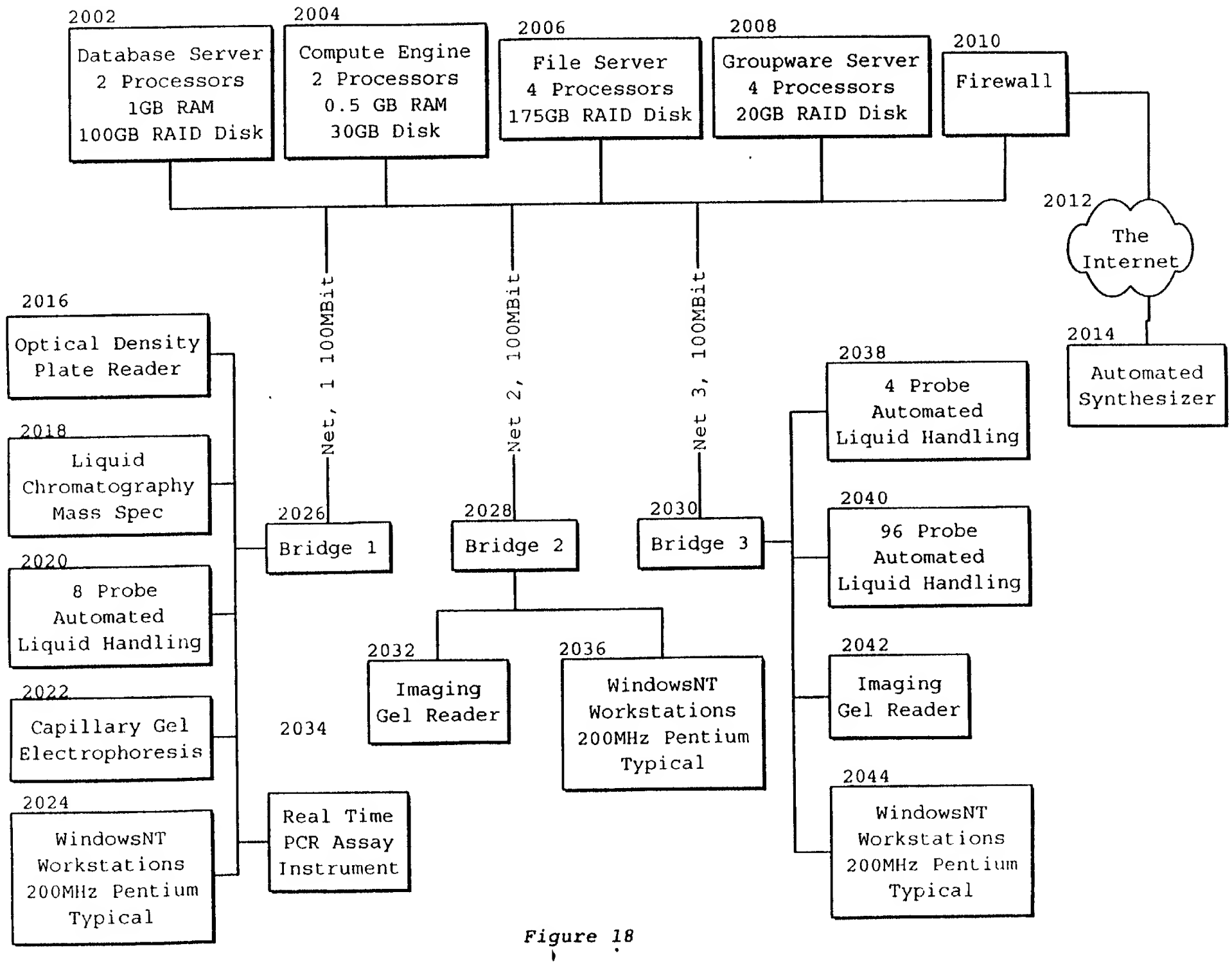

2002
Database Server
2 Processors
1GB RAM
100GB RAID Disk
2004
Compute Engine
2 Processors
0.5 GB RAM
30GB Disk
2006
File Server
4 Processors
175GB RAID Disk
2008
Groupware Server
4 Processors
20GB RAID Disk
2010
Firewall
2012
The Internet
2014
Automated Synthesizer
Net, 1 100MBit
Net 2, 100MBit
Net 3, 100MBit
2016
Optical Density Plate Reader
2018
Liquid Chromatography Mass Spec
2020
8 Probe Automated Liquid Handling
2022
Capillary Gel Electrophoresis
2024
WindowsNT Workstations 200MHz Pentium Typical
2026
Bridge 1
2028
Bridge 2
2030
Bridge 3
2032
Imaging Gel Reader
2034
Real Time PCR Assay Instrument
2036
WindowsNT Workstations 200MHz Pentium Typical
2038
4 Probe Automated Liquid Handling
2040
96 Probe Automated Liquid Handling
2042
Imaging Gel Reader
2044
WindowsNT Workstations 200MHz Pentium Typical

Figure 18

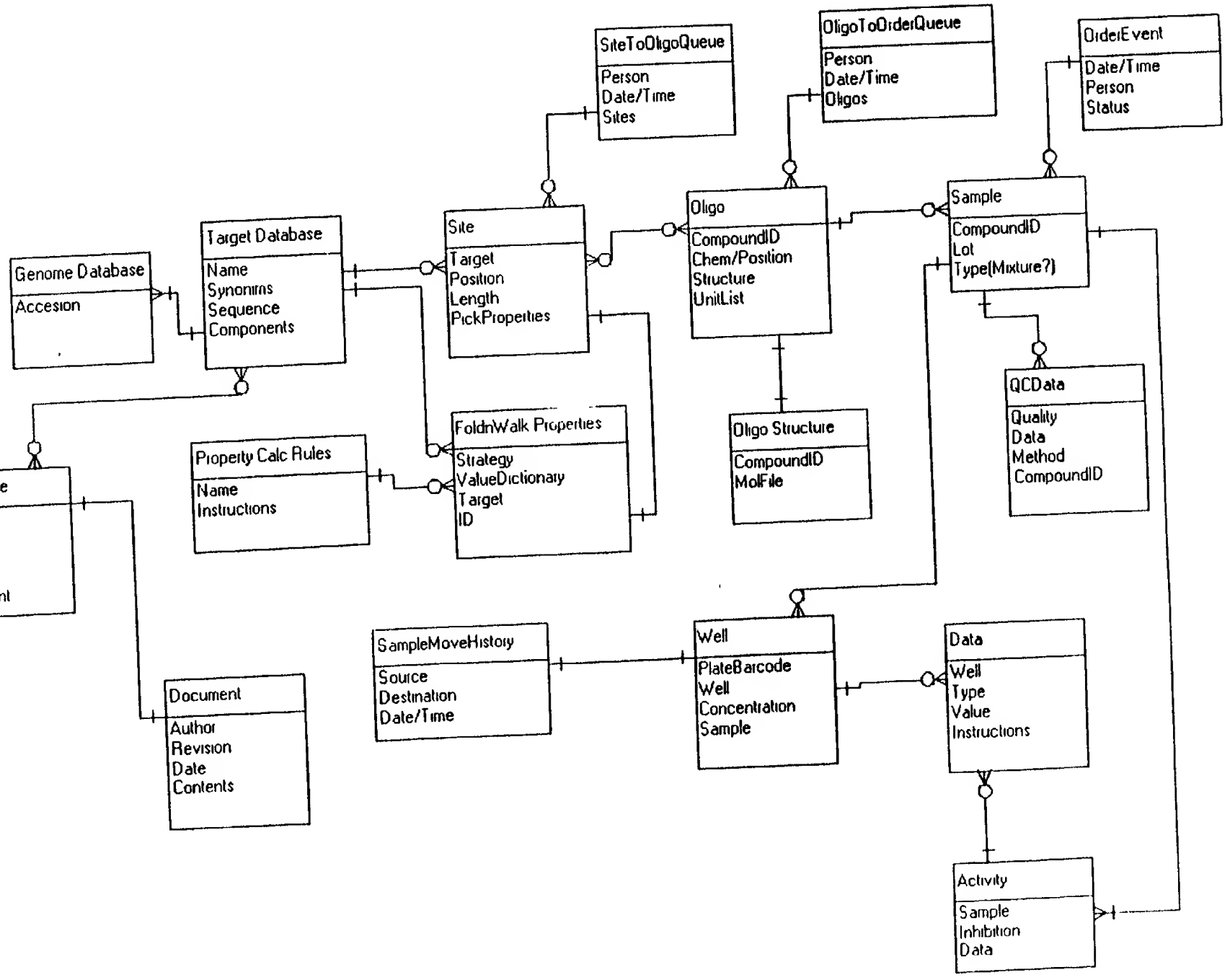

SiteToOligoQueue
Person
Date/Time
Sites
OligoToOrderQueue
Person
Date/Time
Oligos
OrderEvent
Date/Time
Person
Status
Genome Database
Accesion
Target Database
Name
Synonims
Sequence
Components
Site
Target
Position
Length
PickProperties
Oligo
CompoundID
Chem/Position
Structure
UnitList
Sample
CompoundID
Lot
Type(Mixture?)
QCData
Quality
Data
Method
CompoundID
Property Calc Rules
Name
Instructions
FoldnWalk Properties
Strategy
ValueDictionary
Target
ID
Oligo Structure
CompoundID
MolFile
Reference
Journal
Date
Author
Title
Document
Document
Author
Revision
Date
Contents
SampleMoveHistory
Source
Destination
Date/Time
Well
PlateBarcode
Well
Concentration
Sample
Data
Well
Type
Value
Instructions
Activity
Sample
Inhibition
Data

Figure 19

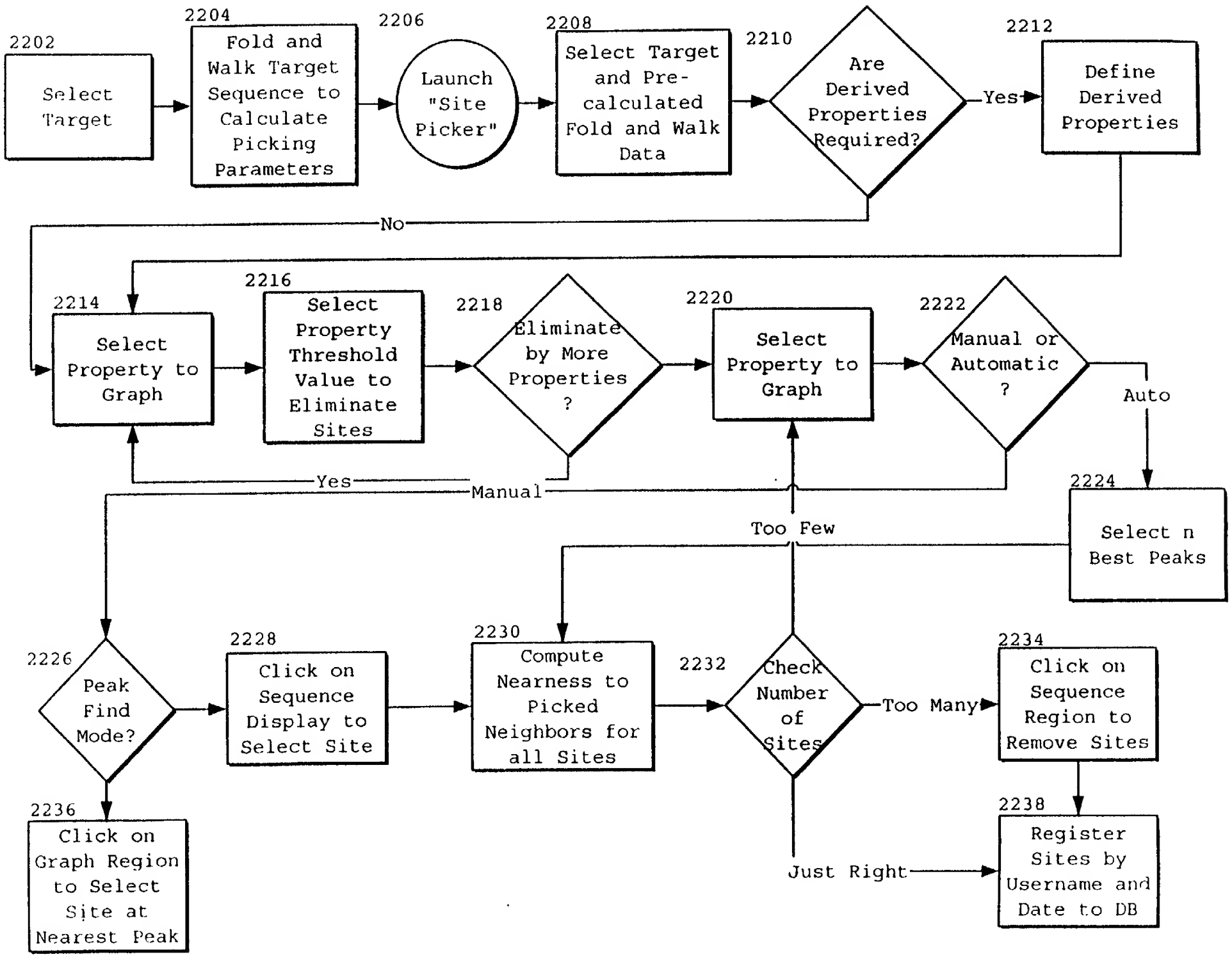
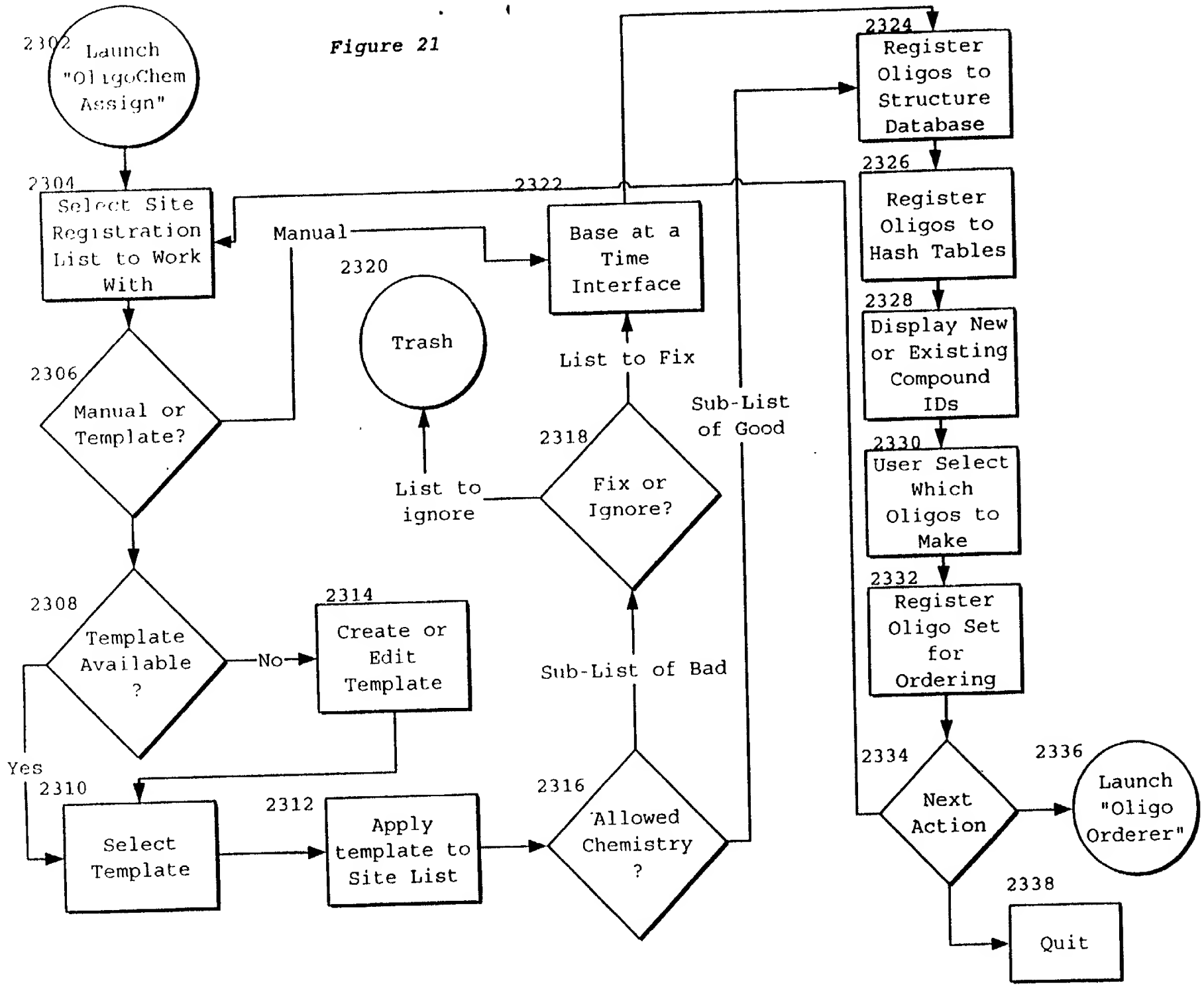
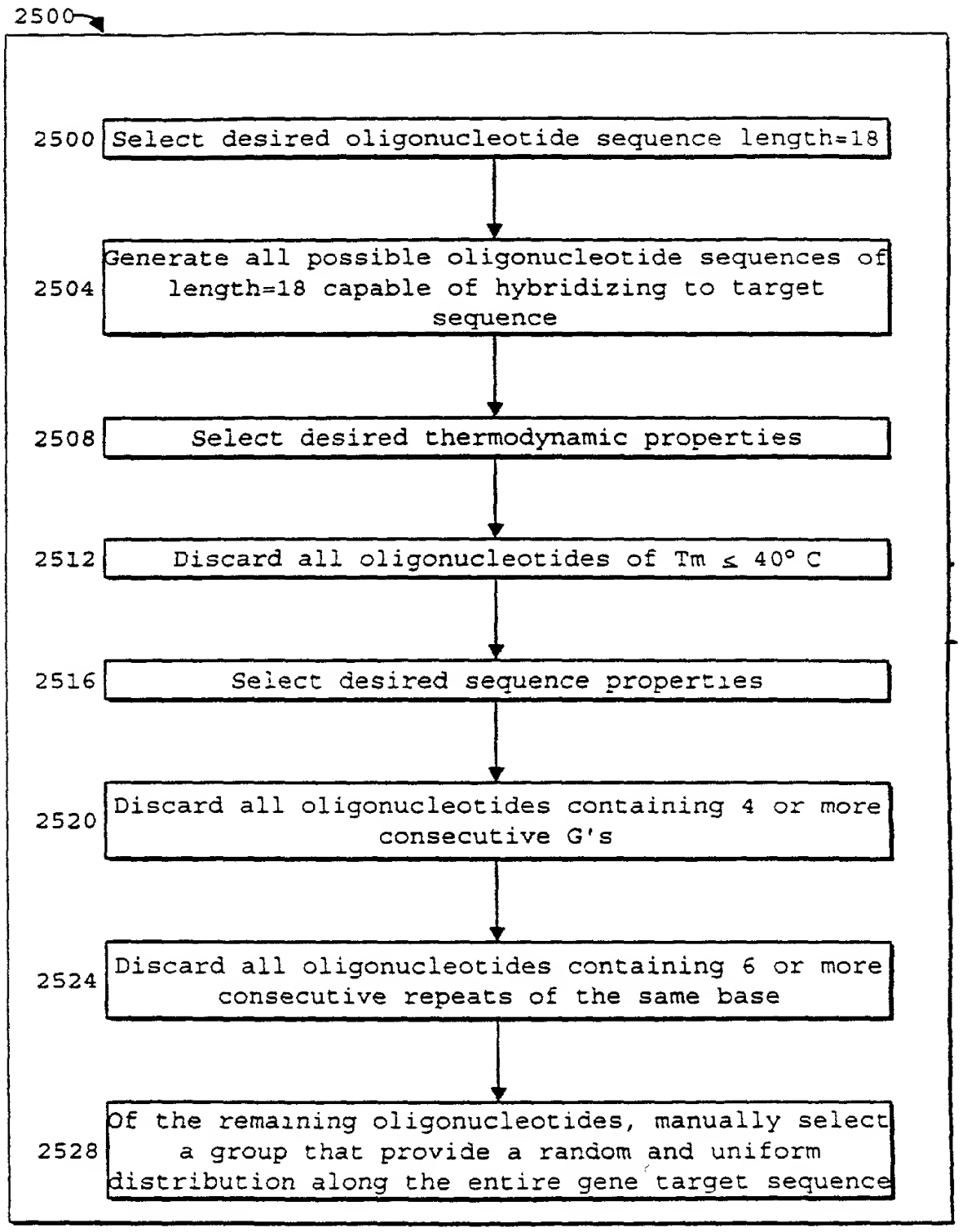

2202
Select Target
2204
Fold and Walk Target Sequence to Calculate Picking Parameters
2206
Launch "Site Picker"
2208
Select Target and Pre-calculated Fold and Walk Data
2210
Are Derived Properties Required?
Yes
2212
Define Derived Properties
No
2214
Select Property to Graph
2216
Select Property Threshold Value to Eliminate Sites
2218
Eliminate by More Properties ?
Yes
2220
Select Property to Graph
Manual
2222
Manual or Automatic ?
Auto
2224
Select n Best Peaks
Too Few
2226
Peak Find Mode?
2228
Click on Sequence Display to Select Site
2230
Compute Nearness to Picked Neighbors for all Sites
2232
Check Number of Sites
Too Many
2234
Click on Sequence Region to Remove Sites
2236
Click on Graph Region to Select Site at Nearest Peak
Just Right
2238
Register Sites by Username and Date to DB

Figure 20

Figure 21


2302
Launch "OligoChem Assign"
2304
Select Site Registration List to Work With
2306
Manual or Template?
2308
Template Available ?
No
2314
Create or Edit Template
Yes
2310
Select Template
2312
Apply template to Site List
2316
Allowed Chemistry ?
Sub-List of Bad
2318
Fix or Ignore?
List to ignore
2320
Trash
Manual
2322
Base at a Time Interface
List to Fix
Sub-List of Good
2324
Register Oligos to Structure Database
2326
Register Oligos to Hash Tables
2328
Display New or Existing Compound IDs
2330
User Select Which Oligos to Make
2332
Register Oligo Set for Ordering
2334
Next Action
2336
Launch "Oligo Orderer"
2338
Quit


2500
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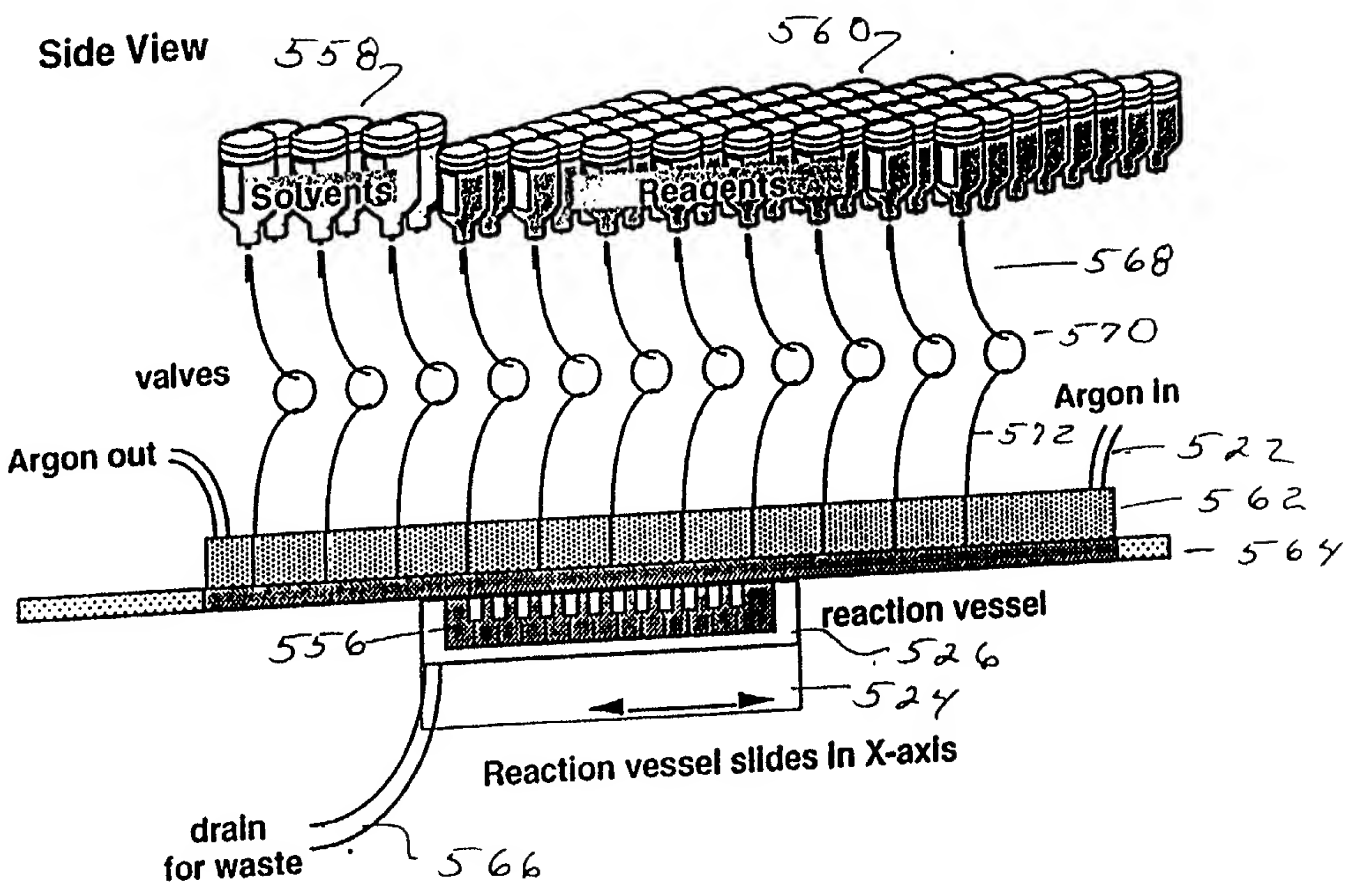
2504 Generate all possible oligonucleotide sequences of length=18 capable of hybridizing to target sequence
2508 Select desired thermodynamic properties
2512 Discard all oligonucleotides of Tm ≤ 40° C
2516 Select desired sequence properties
2520 Discard all oligonucleotides containing 4 or more consecutive G's
2524 Discard all oligonucleotides containing 6 or more consecutive repeats of the same base
2528 Of the remaining oligonucleotides, manually select a group that provide a random and uniform distribution along the entire gene target sequence

Figure 22


Side View
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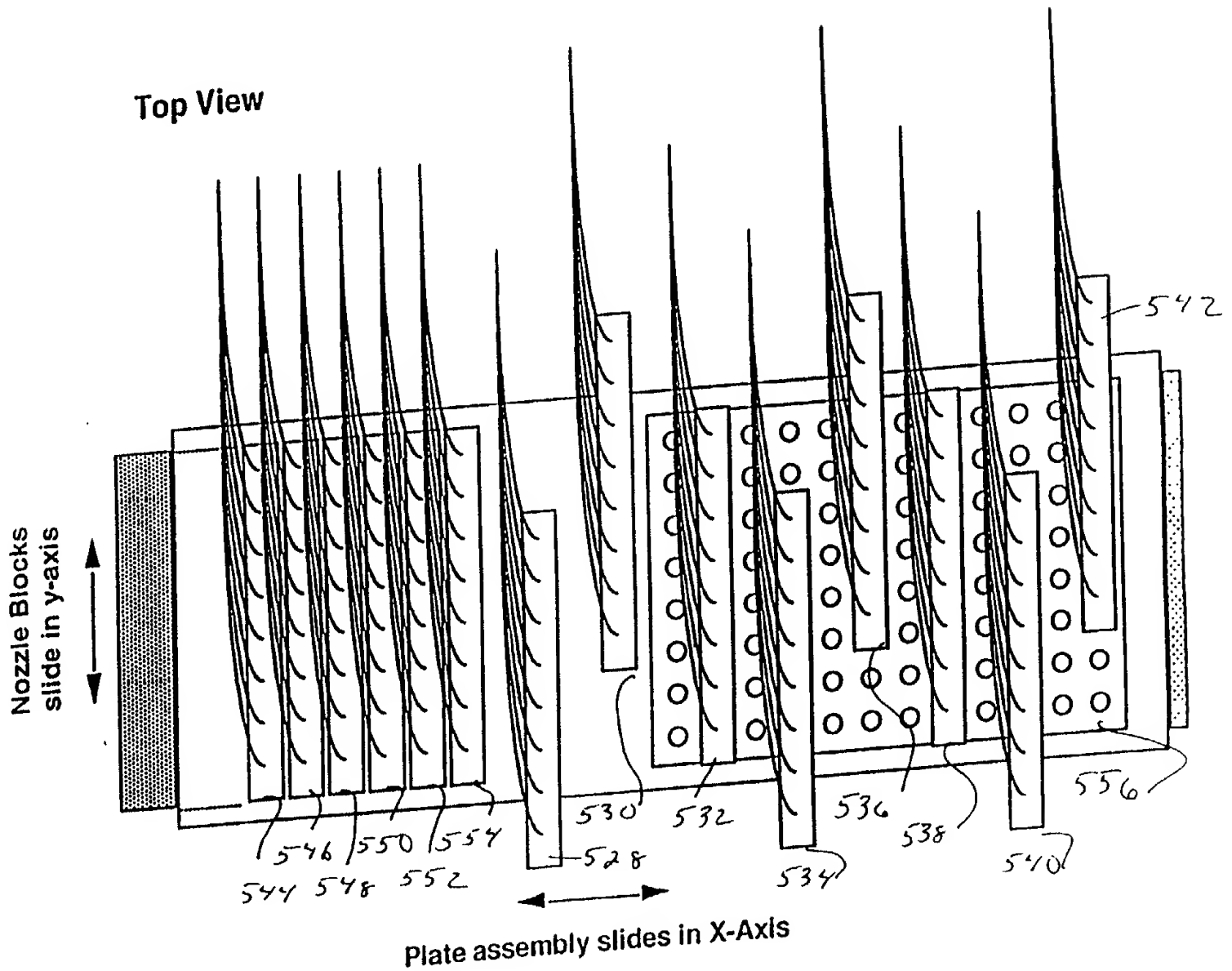
560
Solvents
Reagents
568
570
valves
Argon in
572
522
Argon out
562
564
reaction vessel
556
526
524
Reaction vessel slides in X-axis
drain
for waste
566

Figure 23


Top View
542
Nozzle Blocks slide in y-axis
530
532
536
538
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554
528
544
548
552
534
540
Plate assembly slides in X-Axis

Figure 24

DOCKET NO.: ISIS-3455 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:

Lex M. Cowsert, Brenda F. Baker, John McNeil, Susan M. Freier, Henri M. Sasmor, Douglas G. Brooks, Cara Ohashi, Jacqueline R. Wyatt, Alexander H. Borchers, Timothy A. Vickers

Serial No.: Not Yet Assigned **Group Art Unit:** Not Yet Assigned

Filing Date: Herewith **Examiner:** Not Yet Assigned

For: IDENTIFICATION OF GENETIC TARGETS FOR MODULATION BY OLIGONUCLEOTIDES AND GENERATION OF OLIGONUCLEOTIDES FOR GENE MODULATION

BOX SEQUENCE
Assistant Commissioner for Patents
Washington DC 20231

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 CFR §§ 1.821 THROUGH 1.825

☒ I hereby state, in accordance with the requirements of 37 C.F.R. §1.821(f), that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 CFR §1.821(c) and (e), respectively are the same.

☐ I hereby state that the submission filed in accordance with 37 CFR §1.821(g) does not include new matter.

☐ I hereby state that the submission filed in accordance with 37 CFR §1.821(h) does not include new matter or go beyond the disclosure in the international application as filed.

☐ I hereby state that the amendments, made in accordance with 37 CFR §1.825(a), included in the substitute sheet(s) of the Sequence Listing are supported in the application, as filed, at pages _____. I hereby state that the substitute sheet(s) of the Sequence Listing does (do) not include new matter.

☐ I hereby state that the substitute copy of the computer readable form, submitted in accordance with 37 CFR §1.825(b), is the same as the amended Sequence Listing.

☐ I hereby state that the substitute copy of the computer readable form, submitted in accordance with 37 CFR §1.825(d), contains identical data to that originally filed.

Date: April 13, 1999

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DOCKET NO.: ISIS-3455 PATENT

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **IDENTIFICATION OF GENETIC TARGETS FOR MODULATION BY OLIGONUCLEOTIDES AND GENERATION OF OLIGONUCLEOTIDES FOR GENE MODULATION** the specification of which:

(XX) is attached hereto.

( ) was filed on _____ as Application Serial No. ___ ___ and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	**Number**	**Date Filed**	**Priority Claimed**
_____	_____	_____	___
_____	_____	_____	___
_____	_____	_____	___

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner

provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	**Filing Date**	**Status (patented, pending)**
09/067,638	April 28, 1998	Pending
60/081,483	April 13, 1998	Pending

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: **John W. Caldwell** and **Paul K. Legaard**, Registration Nos. **28,937** and **38,534** of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, and **Herb Boswell, Laurel Bernstein and Henry Wu**, Registration Nos. **27,311, 37,280 and 44,412**, of **ISIS Pharmaceuticals**, 2292 Faraday Avenue, Carlsbad, California 92008.

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Paul K. Legaard
WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP
One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No. **215-568-3100.**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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	Residence: 2395-J Caringa Way Carlsbad, California 92009	Citizenship: United States	
	Post Office Address: Carlsbad, California, 92009		
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	Residence: 427 Retaheim Way La Jolla, California 92037	Citizenship:United States	
	Post Office Address: La Jolla, California 92037		
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	Residence:2946 Renault Street San Diego, California 92122	Citizenship: United States	
	Post Office Address: San Diego, California 92122		
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	Residence: 1751 Orange Blossom Way Encinitas, California 92024	Citizenship:United States	
	Post Office Address: Encinitas, California 92024		

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	Post Office Address: Cardiff, California 92007		
8	Full Name: Jacqueline R. Wyatt	Inventor's Signature:	Date
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	Post Office Address: Encinitas, California 92024		
9	Full Name: Alexander H. Borchers	Inventor's Signature:	Date
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	Post Office Address: Encinitas, California 92024		
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SEQUENCE LISTING

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McNeil, John
Freier, Susan M.
Sasmor, Henri M.
Brooks, Douglas G.
Ohashi, Cara
Wyatt, Jacqueline R.
Borchers, Alexander
Vickers, Timothy A.

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Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His Lys Tyr Cys
70 75 80

gac ccc aac cta ggg ctt cgg gtc cag cag aag ggc acc tca gaa aca 344
Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr Ser Glu Thr
85 90 95

gac acc atc tgc acc tgt gaa gaa ggc tgg cac tgt acg agt gag gcc 392
Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr Ser Glu Ala
100 105 110 115

tgt gag agc tgt gtc ctg cac cgc tca tgc tcg ccc ggc ttt ggg gtc 440
Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly Phe Gly Val
120 125 130

aag cag att gct aca ggg gtt tct gat acc atc tgc gag ccc tgc cca 488
Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu Pro Cys Pro
135 140 145

gtc ggc ttc ttc tcc aat gtg tca tct gct ttc gaa aaa tgt cac cct 536
Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys Cys His Pro
150 155 160

tgg aca agc tgt gag acc aaa gac ctg gtt gtg caa cag gca ggc aca 584
Trp Thr Ser Cys Glu Thr Lys Asp Leu Val Val Gln Gln Ala Gly Thr
165 170 175

aac aag act gat gtt gtc tgt ggt ccc cag gat cgg ctg aga gcc ctg 632
Asn Lys Thr Asp Val Val Cys Gly Pro Gln Asp Arg Leu Arg Ala Leu
180 185 190 195

gtg gtg atc ccc atc atc ttc ggg atc ctg ttt gcc atc ctc ttg gtg 680
Val Val Ile Pro Ile Ile Phe Gly Ile Leu Phe Ala Ile Leu Leu Val
200 205 210

ctg gtc ttt atc aaa aag gtg gcc aag aag cca acc aat aag gcc ccc 728
Leu Val Phe Ile Lys Lys Val Ala Lys Lys Pro Thr Asn Lys Ala Pro
215 220 225

cac ccc aag cag gaa ccc cag gag atc aat ttt ccc gac gat ctt cct 776
His Pro Lys Gln Glu Pro Gln Glu Ile Asn Phe Pro Asp Asp Leu Pro
230 235 240

ggc tcc aac act gct gct cca gtg cag gag act tta cat gga tgc caa 824
Gly Ser Asn Thr Ala Ala Pro Val Gln Glu Thr Leu His Gly Cys Gln
245 250 255

ccg gtc acc cag gag gat ggc aaa gag agt cgc atc tca gtg cag gag 872
Pro Val Thr Gln Glu Asp Gly Lys Glu Ser Arg Ile Ser Val Gln Glu
260 265 270 275

aga cag tga ggctgcaccc acccaggagt gtggccacgt gggcaaacag 921
Arg Gln

gcagttggcc agagagcctg gtgctgctgc tgcaggggtg caggcagaag cggggagcta 981

tgcccagtca gtgccagccc ctc 1004

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<223> PCR Primer

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<210> 87
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<223> PCR Primer

<400> 87
ggtggcagtg tgtctctctg ttc 23

<210> 88
<211> 25
<212> DNA
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<223> PCR Probe

<400> 88
ttccttgcgg tgaaagcgaa ttcct 25

<210> 89
<211> 19
<212> DNA
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<223> PCR Primer

<400> 89
gaaggtgaag gtcggagtc 19

<210> 90
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<223> PCR Primer

<400> 90
gaagatggtg atgggatttc 20

<210> 91
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<223> PCR Probe

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caagcttccc gttctcagcc 20

<210> 92
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<210> 93
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gaacagcact gactgttt 18

<210> 94
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<400> 94
agaacagcac tgactgtt 18

<210> 95
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aagaacagca ctgactgt 18

<210> 96

<211> 18
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<400> 96
aaagaacagc actgactg 18

<210> 97
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caaagaacag cactgact 18

<210> 98
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<210> 99

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<400> 106
ctggctggca caaagaac 18

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ccggctggag cccacc atg gct gca atc cga aag aag ctg gtg atc gtt 109

Met Ala Ala Ile Arg Lys Lys Leu Val Ile Val
1 5 10

ggg gat ggt gcc tgt ggg aag acc tgc ctc ctc atc gtc ttc agc aag 157
Gly Asp Gly Ala Cys Gly Lys Thr Cys Leu Leu Ile Val Phe Ser Lys
15 20 25

gat cag ttt ccg gag gtc tac gtc cct act gtc ttt gag aac tat att 205
Asp Gln Phe Pro Glu Val Tyr Val Pro Thr Val Phe Glu Asn Tyr Ile
30 35 40

gcg gac att gag gtg gac ggc aag cag gtg gag ctg gct ctg tgg gac 253
Ala Asp Ile Glu Val Asp Gly Lys Gln Val Glu Leu Ala Leu Trp Asp
45 50 55

aca gca ggg cag gaa gac tat gat cga ctg cgg cct ctc tcc tac ccg 301
Thr Ala Gly Gln Glu Asp Tyr Asp Arg Leu Arg Pro Leu Ser Tyr Pro
60 65 70 75

gac act gat gtc atc ctc atg tgc ttc tcc atc gac agc cct gac agc 349
Asp Thr Asp Val Ile Leu Met Cys Phe Ser Ile Asp Ser Pro Asp Ser
80 85 90

ctg gaa aac att cct gag aag tgg acc cca gag gtg aag cac ttc tgc 397
Leu Glu Asn Ile Pro Glu Lys Trp Thr Pro Glu Val Lys His Phe Cys
95 100 105

ccc aac gtg ccc atc atc ctg gtg ggg aat aag aag gac ctg agg caa 445
Pro Asn Val Pro Ile Ile Leu Val Gly Asn Lys Lys Asp Leu Arg Gln
110 115 120

gac gag cac acc agg aga gag ctg gcc aag atg aag cag gag ccc gtt 493
Asp Glu His Thr Arg Arg Glu Leu Ala Lys Met Lys Gln Glu Pro Val
125 130 135

cgg tct gag gaa ggc cgg gac atg gcg aac cgg atc agt gcc ttt ggc 541
Arg Ser Glu Glu Gly Arg Asp Met Ala Asn Arg Ile Ser Ala Phe Gly
140 145 150 155

tac ctt gag tgc tca gcc aag acc aag gag gga gtg cgg gag gtg ttt 589
Tyr Leu Glu Cys Ser Ala Lys Thr Lys Glu Gly Val Arg Glu Val Phe
160 165 170

gag atg gcc act cgg gct ggc ctc cag gtc cgc aag aac aag cgt cgg 637
Glu Met Ala Thr Arg Ala Gly Leu Gln Val Arg Lys Asn Lys Arg Arg
175 180 185

agg ggc tgt ccc att ctc tga gatcccccca aagggccctt ttcctacatg 688
Arg Gly Cys Pro Ile Leu
190

ccccctccct tcacaggggt acagaaatta tccccctaca accccagcct cctgagggct 748

ccatactgaa ggctccattt tcagttccct cctgcccagg actgcattgt tttctagccc 808

cgaggtgtgg cacgggccct ccctcccagc gctctgggag ccacgcctat gccctgccct 868

tcctcatggg cccctgggga tcttgccccc ttgaccttcc ccaaaggatg gtcacacacc 928

agcactttat acacttctgg ctcacaggaa agtgtctgca gtagggaccc agagtcccag 988

gcccctggag ttgtttctgc aggggccttg tctctcactg catttggtca ggggggcatg 1048

aataaaggct 1058

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<210> 115
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<223> PCR Primer

<400> 115
ccaggatgat gggcacgtt 19

<210> 116
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<223> PCR Probe

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<210> 117
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atgaggaggc aggtcttc 18

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<210> 155
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<400> 156

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tttaaaatag ccatcttaga atcagtgaaa tatggtaatg tattattttc ctcctttgag 180

ttaggtcttg tgcttttttt tcctggccac taaatttcac aatttccaaa aagcaaaata 240

aacatattct gaatattttt gctgtgaaac acttgacagc agagctttcc accatgaaaa 300

gaagcttcat gagtcacaca ttacatcttt gggttgattg aatgccactg aaacattcta 360

gtagcctgga gaagttgacc tacctgtgga gatgcctgcc attaaatggc atcctgatgg 420

cttaatacac atcactcttc tgtgaagggt tttaattttc aacacagctt actctgtagc 480

atcatgttta cattgtatgt ataaagatta tacaaaggtg caattgtgta tttcttcctt 540

aaaatgtatc agtataggat ttagaatctc catgttgaaa ctctaaatgc atagaaataa 600

aaataataaa aaatttttca ttttggcttt tcagcctagt attaaaactg ataaaagcaa 660

agccatgcac aaaactacct ccctagagaa aggctagtcc cttttcttcc ccattcattt 720

catt atg aac ata gta gaa aac agc ata ttc tta tca aat ttg atg 766
Met Asn Ile Val Glu Asn Ser Ile Phe Leu Ser Asn Leu Met
1 5 10

aaa agc gcc aac acg ttt gaa ctg aaa tac gac ttg tca tgt gaa ctg 814
Lys Ser Ala Asn Thr Phe Glu Leu Lys Tyr Asp Leu Ser Cys Glu Leu
15 20 25 30

tac cga atg tct acg tat tcc act ttt cct gct ggg gtt cct gtc tca 862
Tyr Arg Met Ser Thr Tyr Ser Thr Phe Pro Ala Gly Val Pro Val Ser
35 40 45

gaa agg agt ctt gct cgt gct ggt ttc tat tac act ggt gtg aat gac 910
Glu Arg Ser Leu Ala Arg Ala Gly Phe Tyr Tyr Thr Gly Val Asn Asp
50 55 60

aag gtc aaa tgc ttc tgt tgt ggc ctg atg ctg gat aac tgg aaa aga 958
Lys Val Lys Cys Phe Cys Cys Gly Leu Met Leu Asp Asn Trp Lys Arg
65 70 75

gga gac agt cct act gaa aag cat aaa aag ttg tat cct agc tgc aga 1006
Gly Asp Ser Pro Thr Glu Lys His Lys Lys Leu Tyr Pro Ser Cys Arg
80 85 90

ttc gtt cag agt cta aat tcc gtt aac aac ttg gaa gct acc tct cag 1054
Phe Val Gln Ser Leu Asn Ser Val Asn Asn Leu Glu Ala Thr Ser Gln

95 100 105 110

cct act ttt cct tct tca gta aca aat tcc aca cac tca tta ctt ccg 1102
Pro Thr Phe Pro Ser Ser Val Thr Asn Ser Thr His Ser Leu Leu Pro
115 120 125

ggt aca gaa aac agt gga tat ttc cgt ggc tct tat tca aac tct cca 1150
Gly Thr Glu Asn Ser Gly Tyr Phe Arg Gly Ser Tyr Ser Asn Ser Pro
130 135 140

tca aat cct gta aac tcc aga gca aat caa gat ttt tct gcc ttg atg 1198
Ser Asn Pro Val Asn Ser Arg Ala Asn Gln Asp Phe Ser Ala Leu Met
145 150 155

aga agt tcc tac cac tgt gca atg aat aac gaa aat gcc aga tta ctt 1246
Arg Ser Ser Tyr His Cys Ala Met Asn Asn Glu Asn Ala Arg Leu Leu
160 165 170

act ttt cag aca tgg cca ttg act ttt ctg tcg cca aca gat ctg gca 1294
Thr Phe Gln Thr Trp Pro Leu Thr Phe Leu Ser Pro Thr Asp Leu Ala
175 180 185 190

aaa gca ggc ttt tac tac ata gga cct gga gac aga gtg gct tgc ttt 1342
Lys Ala Gly Phe Tyr Tyr Ile Gly Pro Gly Asp Arg Val Ala Cys Phe
195 200 205

gcc tgt ggt gga aaa ttg agc aat tgg gaa ccg aag gat aat gct atg 1390
Ala Cys Gly Gly Lys Leu Ser Asn Trp Glu Pro Lys Asp Asn Ala Met
210 215 220

tca gaa cac ctg aga cat ttt ccc aaa tgc cca ttt ata gaa aat cag 1438
Ser Glu His Leu Arg His Phe Pro Lys Cys Pro Phe Ile Glu Asn Gln
225 230 235

ctt caa gac act tca aga tac aca gtt tct aat ctg agc atg cag aca 1486
Leu Gln Asp Thr Ser Arg Tyr Thr Val Ser Asn Leu Ser Met Gln Thr
240 245 250

cat gca gcc cgc ttt aaa aca ttc ttt aac tgg ccc tct agt gtt cta 1534
His Ala Ala Arg Phe Lys Thr Phe Phe Asn Trp Pro Ser Ser Val Leu
255 260 265 270

gtt aat cct gag cag ctt gca agt gcg ggt ttt tat tat gtg ggt aac 1582
Val Asn Pro Glu Gln Leu Ala Ser Ala Gly Phe Tyr Tyr Val Gly Asn
275 280 285

agt gat gat gtc aaa tgc ttt tgc tgt gat ggt gga ctc agg tgt tgg 1630
Ser Asp Asp Val Lys Cys Phe Cys Cys Asp Gly Gly Leu Arg Cys Trp
290 295 300

gaa tct gga gat gat cca tgg gtt caa cat gcc aag tgg ttt cca agg 1678
Glu Ser Gly Asp Asp Pro Trp Val Gln His Ala Lys Trp Phe Pro Arg
305 310 315

tgt gag tac ttg ata aga att aaa gga cag gag ttc atc cgt caa gtt 1726
Cys Glu Tyr Leu Ile Arg Ile Lys Gly Gln Glu Phe Ile Arg Gln Val
320 325 330

caa gcc agt tac cct cat cta ctt gaa cag ctg cta tcc aca tca gac 1774
Gln Ala Ser Tyr Pro His Leu Leu Glu Gln Leu Leu Ser Thr Ser Asp
335 340 345 350

agc cca gga gat gaa aat gca gag tca tca att atc cat ttt gaa cct 1822
Ser Pro Gly Asp Glu Asn Ala Glu Ser Ser Ile Ile His Phe Glu Pro
355 360 365

gga gaa gac cat tca gaa gat gca atc atg atg aat act cct gtg att 1870
Gly Glu Asp His Ser Glu Asp Ala Ile Met Met Asn Thr Pro Val Ile
370 375 380

aat gct gcc gtg gaa atg ggc ttt agt aga agc ctg gta aaa cag aca 1918
Asn Ala Ala Val Glu Met Gly Phe Ser Arg Ser Leu Val Lys Gln Thr
385 390 395

gtt caa aga aaa atc cta gca act gga gag aat tat aga cta gtc aat 1966
Val Gln Arg Lys Ile Leu Ala Thr Gly Glu Asn Tyr Arg Leu Val Asn
400 405 410

gat ctt gtg tta gac tta ctc aat gca gaa gat gaa ata agg gaa gag 2014
Asp Leu Val Leu Asp Leu Leu Asn Ala Glu Asp Glu Ile Arg Glu Glu
415 420 425 430

gag aga gaa aga gca act gag gaa aaa gaa tca aat gat tta tta tta 2062
Glu Arg Glu Arg Ala Thr Glu Glu Lys Glu Ser Asn Asp Leu Leu Leu
435 440 445

atc cgg aag aat aga atg gca ctt ttt caa cat ttg act tgt gta att 2110
Ile Arg Lys Asn Arg Met Ala Leu Phe Gln His Leu Thr Cys Val Ile
450 455 460

cca atc ctg gat agt cta cta act gcc gga att att aat gaa caa gaa 2158
Pro Ile Leu Asp Ser Leu Leu Thr Ala Gly Ile Ile Asn Glu Gln Glu

465 470 475

cat gat gtt att aaa cag aag aca cag acg tct tta caa gca aga gaa 2206
His Asp Val Ile Lys Gln Lys Thr Gln Thr Ser Leu Gln Ala Arg Glu
480 485 490

ctg att gat acg att tta gta aaa gga aat att gca gcc act gta ttc 2254
Leu Ile Asp Thr Ile Leu Val Lys Gly Asn Ile Ala Ala Thr Val Phe
495 500 505 510

aga aac tct ctg caa gaa gct gaa gct gtg tta tat gag cat tta ttt 2302
Arg Asn Ser Leu Gln Glu Ala Glu Ala Val Leu Tyr Glu His Leu Phe
515 520 525

gtg caa cag gac ata aaa tat att ccc aca gaa gat gtt tca gat cta 2350
Val Gln Gln Asp Ile Lys Tyr Ile Pro Thr Glu Asp Val Ser Asp Leu
530 535 540

cca gtg gaa gaa caa ttg cgg aga cta caa gaa gaa aga aca tgt aaa 2398
Pro Val Glu Glu Gln Leu Arg Arg Leu Gln Glu Glu Arg Thr Cys Lys
545 550 555

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Val Cys Met Asp Lys Glu Val Ser Ile Val Phe Ile Pro Cys Gly His
560 565 570

cta gta gta tgc aaa gat tgt gct cct tct tta aga aag tgt cct att 2494
Leu Val Val Cys Lys Asp Cys Ala Pro Ser Leu Arg Lys Cys Pro Ile
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tgt agg agt aca atc aag ggt aca gtt cgt aca ttt ctt tca tga 2539
Cys Arg Ser Thr Ile Lys Gly Thr Val Arg Thr Phe Leu Ser
595 600 605

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acttttatcc taatttggtt tccttaaaat ttttatttat ttacaactca aaaaacattg 2659

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aagagaatga taggcttttg ttcttatgaa cgaaaaagag gtagcactac aaacacaata 2779

ttcaatcaaa atttcagcat tattgaaatt gtaagtgaag taaaacttaa gatatttgag 2839

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cccccgcccc ccggagctgc caacattgcc aacgccaccg ccacgctaca cacagcctca 180

actttcagga gacccgtccg tggccttatt tattccaccc ttcctgtaca tcgtagcgaa 240

tcaatccgtg gcgccgcact cctccgcatc cctctttaac agtacccctg ggatggcgtg 300

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Met Asp Pro Ser Val Thr Leu Trp Gln Phe Leu
1 5 10

ctg cag ctg ctg aga gag caa ggc aat ggc cac atc atc tcc tgg act 396
Leu Gln Leu Leu Arg Glu Gln Gly Asn Gly His Ile Ile Ser Trp Thr

15 20 25

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Ser Arg Asp Gly Gly Glu Phe Lys Leu Val Asp Ala Glu Glu Val Ala
30 35 40

cgg ctg tgg gga cta cgc aag aac aag acc aac atg aat tac gac aag 492
Arg Leu Trp Gly Leu Arg Lys Asn Lys Thr Asn Met Asn Tyr Asp Lys
45 50 55

ctc agc cgg gcc ttg cgg tac tac tat gac aag aac atc atc cgc aag 540
Leu Ser Arg Ala Leu Arg Tyr Tyr Tyr Asp Lys Asn Ile Ile Arg Lys
60 65 70 75

gtg agc ggc cag aag ttc gtc tac aag ttt gtg tcc tac cct gag gtc 588
Val Ser Gly Gln Lys Phe Val Tyr Lys Phe Val Ser Tyr Pro Glu Val
80 85 90

gca ggg tgc tcc act gag gac tgc ccg ccc cag cca gag gtg tct gtt 636
Ala Gly Cys Ser Thr Glu Asp Cys Pro Pro Gln Pro Glu Val Ser Val
95 100 105

acc tcc acc atg cca aat gtg gcc cct gct gct ata cat gcc gcc cca 684
Thr Ser Thr Met Pro Asn Val Ala Pro Ala Ala Ile His Ala Ala Pro
110 115 120

ggg gac act gtc tct gga aag cca ggc aca ccc aag ggt gca gga atg 732
Gly Asp Thr Val Ser Gly Lys Pro Gly Thr Pro Lys Gly Ala Gly Met
125 130 135

gca ggc cca ggc ggt ttg gca cgc agc agc cgg aac gag tac atg cgc 780
Ala Gly Pro Gly Gly Leu Ala Arg Ser Ser Arg Asn Glu Tyr Met Arg
140 145 150 155

tcg ggc ctc tat tcc acc ttc acc atc cag tct ctg cag ccg cag cca 828
Ser Gly Leu Tyr Ser Thr Phe Thr Ile Gln Ser Leu Gln Pro Gln Pro
160 165 170

ccc cct cat cct cgg cct gct gtg gtg ctc ccc aat gca gct cct gca 876
Pro Pro His Pro Arg Pro Ala Val Val Leu Pro Asn Ala Ala Pro Ala
175 180 185

ggg gca gca gcg ccc ccc tcg ggg agc agg agc acc agt cca agc ccc 924
Gly Ala Ala Ala Pro Pro Ser Gly Ser Arg Ser Thr Ser Pro Ser Pro
190 195 200

ttg gag gcc tgt ctg gag gct gaa gag gcc ggc ttg cct ctg cag gtc 972
Leu Glu Ala Cys Leu Glu Ala Glu Glu Ala Gly Leu Pro Leu Gln Val
205 210 215

atc ctg acc ccg ccc gag gcc cca aac ctg aaa tcg gaa gag ctt aat 1020
Ile Leu Thr Pro Pro Glu Ala Pro Asn Leu Lys Ser Glu Glu Leu Asn
220 225 230 235

gtg gag ccg ggt ttg ggc cgg gct ttg ccc cca gaa gtg aaa gta gaa 1068
Val Glu Pro Gly Leu Gly Arg Ala Leu Pro Pro Glu Val Lys Val Glu
240 245 250

ggg ccc aag gaa gag ttg gaa gtt gcg ggg gag aga ggg ttt gtg cca 1116
Gly Pro Lys Glu Glu Leu Glu Val Ala Gly Glu Arg Gly Phe Val Pro
255 260 265

gaa acc acc aag gcc gag cca gaa gtc cct cca cag gag ggc gtg cca 1164
Glu Thr Thr Lys Ala Glu Pro Glu Val Pro Pro Gln Glu Gly Val Pro
270 275 280

gcc cgg ctg ccc gcg gtt gtt atg gac acc gca ggg cag gcg ggc ggc 1212
Ala Arg Leu Pro Ala Val Val Met Asp Thr Ala Gly Gln Ala Gly Gly
285 290 295

cat gcg gct tcc agc cct gag atc tcc cag ccg cag aag ggc cgg aag 1260
His Ala Ala Ser Ser Pro Glu Ile Ser Gln Pro Gln Lys Gly Arg Lys
300 305 310 315

ccc cgg gac cta gag ctt cca ctc agc ccg agc ctg cta ggt ggg ccg 1308
Pro Arg Asp Leu Glu Leu Pro Leu Ser Pro Ser Leu Leu Gly Gly Pro
320 325 330

gga ccc gaa cgg acc cca gga tcg gga agt ggc tcc ggc ctc cag gct 1356
Gly Pro Glu Arg Thr Pro Gly Ser Gly Ser Gly Ser Gly Leu Gln Ala
335 340 345

ccg ggg ccg gcg ctg acc cca tcc ctg ctt cct acg cat aca ttg acc 1404
Pro Gly Pro Ala Leu Thr Pro Ser Leu Leu Pro Thr His Thr Leu Thr
350 355 360

ccg gtg ctg ctg aca ccc agc tcg ctg cct cct agc att cac ttc tgg 1452
Pro Val Leu Leu Thr Pro Ser Ser Leu Pro Pro Ser Ile His Phe Trp
365 370 375

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Ser Thr Leu Ser Pro Ile Ala Pro Arg Ser Pro Ala Lys Leu Ser Phe

380 385 390 395

cag ttt cca tcc agt ggc agc gcc cag gtg cac atc cct tct atc agc 1548
Gln Phe Pro Ser Ser Gly Ser Ala Gln Val His Ile Pro Ser Ile Ser
400 405 410

gtg gat ggc ctc tcg acc ccc gtg gtg ctc tcc cca ggg ccc cag aag 1596
Val Asp Gly Leu Ser Thr Pro Val Val Leu Ser Pro Gly Pro Gln Lys
415 420 425

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ttacacgtct cctacttcaa tggtaggggc ggtttattta tttatttttt gaaggccact 1892

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cagcctgccc ttggggagat gagggagccc tgtctgcgtt tttggatgtg agtagaagag 2072

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Glu Ser Lys Arg Ile Asn Ala Glu Ile Glu Lys Gln Leu Arg Arg Asp
20 25 30

aag cgc gac gcc cgg cgc gag ctc aag ctg ctg ctg ctc ggc acg ggc 144
Lys Arg Asp Ala Arg Arg Glu Leu Lys Leu Leu Leu Leu Gly Thr Gly
35 40 45

gag agc ggg aag agc acg ttc atc aag cag atg cgc atc atc cac ggc 192
Glu Ser Gly Lys Ser Thr Phe Ile Lys Gln Met Arg Ile Ile His Gly
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Gln Asn Ile Phe Thr Ala Met Gln Ala Met Ile Arg Ala Met Glu Thr
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Leu Lys Ile Leu Tyr Lys Tyr Glu Gln Asn Lys Ala Asn Ala Leu Leu
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Ile Arg Glu Val Asp Val Glu Lys Val Thr Thr Phe Glu His Gln Tyr
115 120 125

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Val Ser Ala Ile Lys Thr Leu Trp Glu Asp Pro Gly Ile Gln Glu Cys
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Tyr Asp Arg Arg Arg Glu Tyr Gln Leu Ser Asp Ser Ala Lys Tyr Tyr
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ctg acc gac gtt gac cgc atc gcc acc ttg ggc tac ctg ccc acc cag 528
Leu Thr Asp Val Asp Arg Ile Ala Thr Leu Gly Tyr Leu Pro Thr Gln
165 170 175

cag gac gtg ctg cgg gtc cgc gtg ccc acc acc ggc atc atc gag tac 576
Gln Asp Val Leu Arg Val Arg Val Pro Thr Thr Gly Ile Ile Glu Tyr
180 185 190

cct ttc gac ctg gag aac atc atc ttc cgg atg gtg gat gtg ggg ggc 624
Pro Phe Asp Leu Glu Asn Ile Ile Phe Arg Met Val Asp Val Gly Gly
195 200 205

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Gln Arg Ser Glu Arg Arg Lys Trp Ile His Cys Phe Glu Asn Val Thr
210 215 220

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Ser Ile Met Phe Leu Val Ala Leu Ser Glu Tyr Asp Gln Val Leu Val
225 230 235 240

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Glu Ser Asp Asn Glu Asn Arg Met Glu Glu Ser Lys Ala Leu Phe Arg
245 250 255

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Thr Ile Ile Thr Tyr Pro Trp Phe Gln Asn Ser Ser Val Ile Leu Phe
260 265 270

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Leu Asn Lys Lys Asp Leu Leu Glu Asp Lys Ile Leu Tyr Ser His Leu
275 280 285

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Val Asp Tyr Phe Pro Glu Phe Asp Gly Pro Gln Arg Asp Ala Gln Ala
290 295 300

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Ala Arg Glu Phe Ile Pro Lys Met Phe Val Asp Leu Asn Pro Asp Ser
305 310 315 320

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Asp Lys Ile Ile Tyr Ser His Phe Thr Cys Ala Thr Asp Thr Glu Asn

325 330 335

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Ile Arg Phe Val Phe Ala Ala Val Lys Asp Thr Ile Leu Gln Leu Asn
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gcgggagcct cgggcacc atg agc gac gtg gct att gtg aag gag ggt tgg 231
Met Ser Asp Val Ala Ile Val Lys Glu Gly Trp
1 5 10

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Leu His Lys Arg Gly Glu Tyr Ile Lys Thr Trp Arg Pro Arg Tyr Phe
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Leu Leu Lys Asn Asp Gly Thr Phe Ile Gly Tyr Lys Glu Arg Pro Gln
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Asp Val Asp Gln Arg Glu Ala Pro Leu Asn Asn Phe Ser Val Ala Gln
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Cys Gln Leu Met Lys Thr Glu Arg Pro Arg Pro Asn Thr Phe Ile Ile
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cgc tgc ctg cag tgg acc act gtc atc gaa cgc acc ttc cat gtg gag 471
Arg Cys Leu Gln Trp Thr Thr Val Ile Glu Arg Thr Phe His Val Glu
80 85 90

act cct gag gag cgg gag gag tgg aca acc gcc atc cag act gtg gct 519
Thr Pro Glu Glu Arg Glu Glu Trp Thr Thr Ala Ile Gln Thr Val Ala
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Asp Gly Leu Lys Lys Gln Glu Glu Glu Glu Met Asp Phe Arg Ser Gly

110 115 120

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Lys Pro Lys His Arg Val Thr Met Asn Glu Phe Glu Tyr Leu Lys Leu
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160 165 170

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Thr Gly Arg Tyr Tyr Ala Met Lys Ile Leu Lys Lys Glu Val Ile Val
175 180 185

gcc aag gac gag gtg gcc cac aca ctc acc gag aac cgc gtc ctg cag 807
Ala Lys Asp Glu Val Ala His Thr Leu Thr Glu Asn Arg Val Leu Gln
190 195 200

aac tcc agg cac ccc ttc ctc aca gcc ctg aag tac tct ttc cag acc 855
Asn Ser Arg His Pro Phe Leu Thr Ala Leu Lys Tyr Ser Phe Gln Thr
205 210 215

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His Asp Arg Leu Cys Phe Val Met Glu Tyr Ala Asn Gly Gly Glu Leu
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Phe Phe His Leu Ser Arg Glu Arg Val Phe Ser Glu Asp Arg Ala Arg
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255 260 265

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270 275 280

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285 290 295

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Ile Lys Asp Gly Ala Thr Met Lys Thr Phe Cys Gly Thr Pro Glu Tyr
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Pro Phe Tyr Asn Gln Asp His Glu Lys Leu Phe Glu Leu Ile Leu Met
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415 420 425

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Gln Val Thr Ser Glu Thr Asp Thr Arg Tyr Phe Asp Glu Glu Phe Thr
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445 450 455

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